Emergence, Mechanisms and Clinical Relevance of HIV-1 Drug Resistance in the Era of Combination Antiretroviral Therapy

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Summary

Human immunodeficiency virus (HIV) infections can currently not be cured with antiretroviral drugs. The strategy of the therapy is to inhibit the viral replication with a daily intake of antiretroviral drugs. Thus, viral load can be suppressed to undetectable levels. Long-term viral suppression can be achieved with the simultaneous administration of different drug classes, the so-called combination antiretroviral therapy (cART). If intracellular drug levels decrease, e.g. because of missed drugs or drug-drug interactions, resistance-associated mutations may occur and may lead to a therapy failure.

In this study, we aimed to analyze different aspects of HIV drug resistance by using data from the Swiss HIV Cohort Study (SHCS) and the SHCS drug resistance database. The SHCS is an ongoing, observational database that is recruiting HIV infected patients since 1988. The SHCS drug resistance database is linked to the SHCS and exists since 2001. It includes all HIV sequences from the four authorized laboratories in Switzerland that were generated for the purpose of genotypic resistance testing in the framework of the SHCS.

Etravirine is a new generation nonnucleoside reverse transcriptase inhibitor (NNRTI). It has a different resistance profile than common NNRTIs. Therefore, it is still effective among highly treatment-experienced patients with multi-drug resistant HIV strains. In chapter 1, we analyzed the therapeutic potential of etravirine in the SHCS. We showed that about 10% of treatment-naïve patients had etravirine resistance-associated mutations. These mutations were mainly polymorphisms. Further, we saw that the estimated activity of etravirine is lowest in patients harbouring extensive multi-drug resistant viruses, thus limiting etravirine use in those who are most in need. However, the estimated activity of etravirine varied widely between different interpretation algorithms for genotypic resistance tests.

Raltegravir is also a new antiretroviral drug. It is the first integrase inhibitor (INI) and was registered in 2008 for highly treatment-experienced patients with detectable viral loads. In chapter 2, we demonstrated that raltegravir initially was mainly administered in salvage treatment, whrere no other potent drugs were available or to circumvent a treatment with toxic drugs, such as enfuvirtide. The week 24 efficacy of raltegravir in

our study was comparable to randomized-controlled trials performed by the manufacturer.

To date, the optimal treatment strategy for patients harbouring extensive multi-drug resistant viruses is unknown. Usually, new antiretroviral drugs or antiretroviral drug classes are administered in combination with nucleoside reverse transcriptase inhibitors (NRTI), even if NRTI resistance-associated mutations are present. However, it is unknown whether the administration of these NRTIs results in an additional virological benefit. In chapter 3, we studied highly treatment-experienced patients who initiated a therapy including raltegravir. We found evidence that the administration of partially active or inactive NRTIs improved the viral suppression rate at week 24.

Two genotypic changes exist that reduce the activity of all NRTIs, the 69 insertion and Q151M mutation. In chapter 4, we identified predictors for the emergence of these mutations. We saw that the 69 insertion and Q151M only occurred if patients were previously treated with a mono-/dual-NRTI therapy. Exposure to didanosine was associated with the emergence of 69 insertion. For Q151M, no risk factor was identified. However, patients with Q151M tended to have an increased mortality risk compared to a control group of highly NRTI experienced patients. In chapter 5, we additionally identified polymorphic mutations associated with the emergence of 69 insertion or Q151M. We found evidence that the 69 insertion is the end point of the thymidine-analogue mutation (TAM) 1 pathway. Further, we identified eight polymorphic mutations associated with the emergence of 69 insertion or Q151M.

HIV-1 is subdivided into different subtypes and circulating recombinant forms (CRF). Subtypes and CRFs are determined on the basis of the viral genome and generally originate from distinct geographic areas reflecting different HIV subepidemics. All antiretroviral drugs against HIV were mainly designed and tested in Western countries where subtype B is predominant. A long time, it remained unclear whether antiretroviral drugs have a comparable activity to non-B subtypes. In chapter 6, we showed that the long-term virological outcome was even better among Caucasians infected with non-B subtypes compared to subtype B. In particular, patients infected with CRF02_AG and subtype A had a better long-term virological outcome.

Zusammenfassung

Heute kann eine Infektion mit dem Humanen Immundefizienz-Virus (HIV) mit antiretroviralen Medikamenten nicht geheilt werden. Die Therapie hat zum Ziel, durch tägliche Einnahme von Medikamenten die virale Replikation zu hemmen. Dadurch kann eine undetektierbare Viruslast erreicht werden. Mit den heutigen Kombinationstherapien, die verschiedene Medikamentenklassen enthalten, können langjährige Therapieerfolge erzielt werden. Bei unregelmässiger Einnahme der Medikamente oder wenn die intrazelluläre Medikamentenkonzentrationen aus anderen Gründen zu tief sind, können Resistenzmutationen auftreten, die zu einem Therapieversagen führen.

In dieser Arbeit haben wir verschiedene Aspekte der HIV Resistenz anhand von Daten der *Swiss HIV Cohort Study (SHCS)* und der *SHCS drug resistance database* studiert. Die SHCS ist eine fortlaufende Studie, die seit 1988 HIV Patienten rekrutiert. Die *SHCS drug resistance database* ist eine angekoppelte Datenbank, die seit 2001 besteht. Sie enthält alle HIV Sequenzen, die zwecks genotypischer Resistenztestung in den vier autorisierten Labors in der Schweiz im Rahmen der SHCS gemacht werden.

Etravirine ist ein neuer, nicht-nukleosidanaloger Hemmer der reversen Transkriptase (NNRTI). Etravirine ist wirksam in stark vorbehandelten Patienten, da sich das Resistenzprofil von herkömmlichen NNRTI unterscheidet. In Kapitel 1 haben wir das therapeutische Potential von Etravirine in der SHCS studiert. Wir haben aufgezeigt, dass, zirka 10% der unbehandelten Patienten Viren mit Etravirine-Mutationen vorweisen. Weiter sahen wir, dass die geschätzte Aktivität von Etravirine in Patienten mit den geringsten Therapieoptionen am kleinsten ist, also in den am stärksten vorbehandelten Patienten, welche am meisten auf die Aktivität angewiesen wären. Die geschätzte Aktivität von Etravirine variierte aber stark zwischen verschiedenen Interpretationsalgorithmen von genotypischen Resistenztests.

Raltegravir ist ebenfalls ein neueres Medikament. Es ist der erste Hemmer der Integrase und wurde 2008 für stark vorbehandelte Patienten mit nachweisbarer Viruslast zugelassen. In Kapitel 2 zeigten wir, dass Raltegravir anfänglich vor allem eingesetzt wurde in Patienten, die keine anderen aktiven Medikamente zur Verfügung hatten und um Behandlungen mit toxischen Medikamenten, wie

Enfuvirtide zu umgehen. Die Wirksamkeit von Raltegravir nach 24 Wochen war vergleichbar mit den randomisierten, kontrollierten Studien des Herstellers.

Es gibt immer noch Unklarheiten, wie man stark vorbehandelte Patienten mit vielen Resistenzmutationen am besten therapiert. Oft werden neuere Medikamente verschrieben, aber trotz vorliegender Resistenz zusätzlich noch nukleosidanaloge Hemmer der reversen Transkriptase (NRTI) dazugegeben. Der Nutzen dieser NRTI ist aber unklar. In Kapitel 3 konnten wir nachweisen, dass in stark vorbehandelten Patienten, die Raltegravir erhalten, die Verabreichung solcher NRTI eine positive Auswirkung auf den Therapieerfolg nach 24 Wochen hatte.

Es gibt zwei sehr seltene genotypische Veränderungen, die 69 Insertion und die Q151M Mutation, die die Wirksamkeit von allen NRTIs reduzieren. In Kapitel 4 haben wir Risikofaktoren für das Auftreten dieser Mutationen studiert. Wir zeigten, dass die 69 Insertion und die Q151M Mutation in der SHCS nur vorkamen, wenn die Patienten zuvor alleine mit NRTIs behandelt wurden (nicht in einer Kombinationstherapie). Das Verabreichen von Didanosine war assoziiert mit dem Auftreten von der 69 Insertion. Für Q151M wurde kein Risikofaktor gefunden, aber Patienten mit Q151M hatten tendenziell ein höheres Mortalitätsrisiko als Patienten einer Kontrollgruppe. In Kapitel 5 haben wir zusätzlich analysiert, ob es Polymorphismen gibt, die mit dem Auftreten der 69 Insertion oder Q151M zusammmenhängen. Wir stellten fest, dass die 69 Insertion erst auftrat, nachdem Thymidinanaloge-Mutationen (TAM) 1 entstanden sind. Zudem fanden wir acht Polymorphismen, die möglicherweise das Auftreten der 69 Insertion und Q151M beeinflusst haben.

HIV-1 wird in verschiedene Subtypen und zirkulierende rekombinante Formen (CRFs) unterteilt, die anhand des Genoms unterschieden werden und jeweils gehäuft in bestimmten geographischen Regionen vorkommen und unterschiedliche HIV Subepidemien representieren. Alle antiretroviralen Medikamente gegen HIV wurden hauptsächlich in westlichen Ländern entwickelt und getestet, wo Subtype B-Infektionen am häufigsten vorkommen. Lange wusste man nicht, ob diese Medikamente für Infektionen mit anderen Subtypen dieselbe Wirkung haben. In Kapitel 6 wiesen wir in einer Population von Kaukasiern nach, dass im Vergleich zu Subtyp B die Zeit bis zum Therapieversagen in nicht-Subtyp B infizierten Patienten länger ist. Vor allem CRF02_AG und Subtyp A hatten ein besseres langfristiges Therapieansprechen.

Introduction

1.1 Epidemiology and diversity of HIV

In 1981, physicians in San Francisco firstly described the acquired immunodeficiency syndrome (AIDS) among previously healthy homosexual men.[1-3] It was initially also called gay-related immunodeficiency syndrome (GRID).[4, 5] The cause of AIDS remained unknown until 1983, when the human immunodeficiency virus (HIV), a retrovirus of the lentivirus family, was discovered.[6-9] HIV is transmitted by body fluids, such as blood, semen, breast milk, or vaginal secretions. The main transmission routes of HIV are sexual intercourse, sharing injection paraphernalia and vertical transmission from mother to child, as well as transmission via blood products.[10] In the last 30 years, the HIV epidemic has spread all over the world. In 2009, approximately 33 million people were estimated to be infected with HIV worldwide, approximately 2.6 new HIV infection occurred and 1.8 million people died of AIDS.[11] The prevalence of HIV differs markedly between regions. The highest prevalence is described in Sub-Saharan countries where up to 28% of adults (15-49 years) are infected.[11]

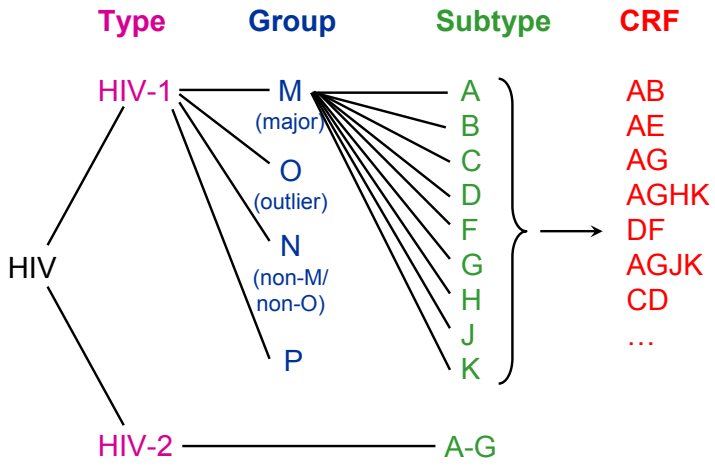

Figure 1. Nomenclature of HIV.

The HIV epidemic is characterized by a large viral diversity that is partially explained by its evolution. HIV entered the human population as a result of zoonotic transmission from primates. There is evidence that such events occurred several times.[12-18] As shown in figure 1, at least two major types of HIV exist, HIV-1 and HIV-2.[19, 20] HIV-1 is more infectious, has a more rapid disease progression and has spread globally,[21] whereas the epidemic of HIV-2 is mainly endemic in Western

Africa. HIV-1 was classified into three groups, the major group M, the outlier group O, and another group N (non-O/non-M). Recently, a new group was described and named P. Viruses from group P are closely related to the gorilla simian immunodeficiency virus.[22, 23] Group M is most common. It is further subdivided into different subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs).[20] CRFs are intersubtype recombinant forms that are spreading in the populations. A new form is defined when at least three people without direct epidemiologic linkage are found to be infected. URFs are intersubtype recombinant forms that were described less often, only once or twice.[20]

In Western countries, subtype B is predominant, but in the last decade the prevalence of non-B subtypes rose also in these regions, in particular in Europe.[24-26] Globally, subtype B causes only about 11% of infections.[27] Worldwide, subtype C is most common, it accounts for nearly half of all infections. Subtype C mainly occurs in Sub-Saharan Africa. Other highly predominant subtypes and CRFs are A (12%), CRF02_AG (8%) and CRR01_AE (5%). Specific subtypes and CRFs are strongly correlated with geographical regions (figure 2).[27]

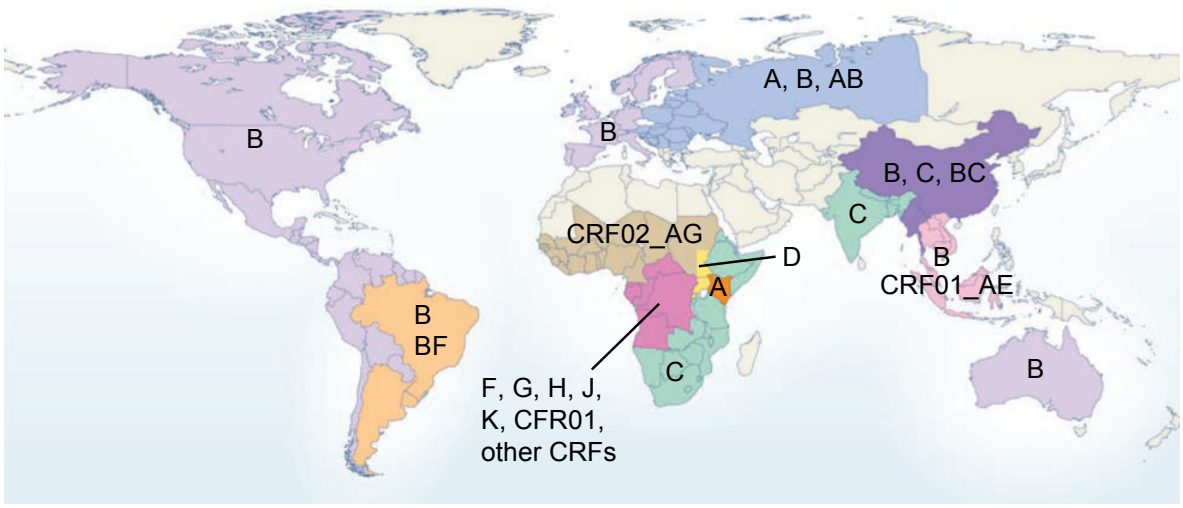

Figure 2. Global distribution of HIV-1 subtypes and circulating recombinant forms. Adapted from [28].

1.2 Viral structure and genome

HIV-1 is a retrovirus with a diameter of about 120 nm. It consists of two single stranded RNAs (figure 3) that are enclosed by the nucleocapsid. The RNA is bound to proteins and important enzymes such as the reverse transcriptase and integrase. A matrix with the viral protein p17 surrounds the capsid and the envelope consists of

a lipid membrane including the glycoproteins gp120 and gp41.[29, 30] The HIV-1 genome is encoded by 9749 nucleotides.[31] It is primarily a coding RNA and contains 9 open reading frames (*gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*) that encode 15 proteins.[30] Four *gag* proteins exist that are essential for the viral structure: matrix, capsid, nucleocapsid and p6. The *pol* region encodes for the following three essential enzymes: the polymerase, reverse transcriptase and integrase. The *env* region encodes for the two surface gp120 and gp41. The six other genes code for regulatory or accessory proteins.[29, 30] Recently, the secondary structure of the entire HIV-1 RNA genome was characterized.[32]

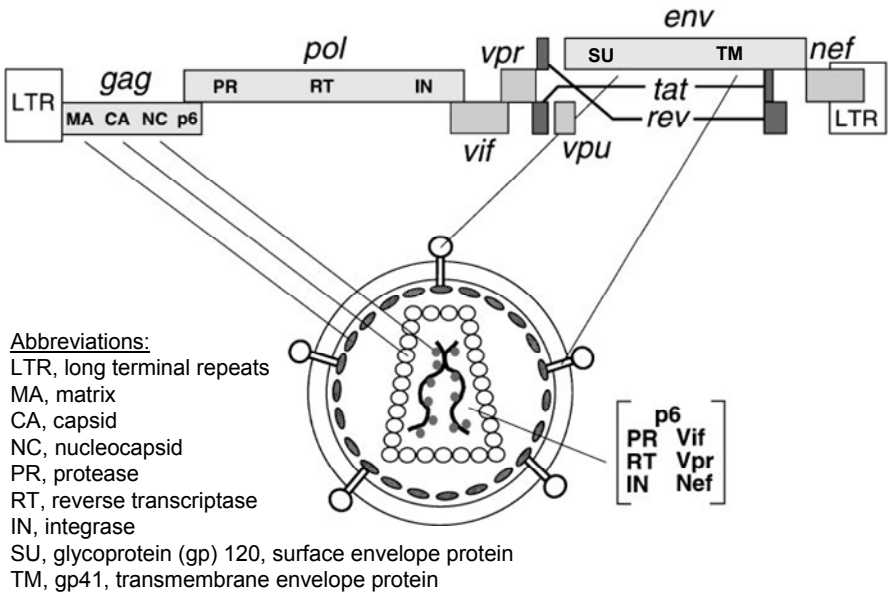

Figure 3. Structure and genome of HIV-1. Adapted from [30].

1.3 Viral life cycle

HIV's most common route of entry in the human body is via the mucosa: vaginal, rectal, and to lesser extent also oral. In these cases, HIV needs to overcome the mucosal barrier to reach its target cells, a step that is not necessary when HIV is transmitted by a direct route (e.g. sharing injection paraphernalia, injuries or blood products).[10] Once HIV entered the body, the initial step of the life cycle is the attachment and binding of the virus to the host cell (figure 4). Mainly CD4+ cells and macrophages are infected by HIV and allow viral replication, but all cells expressing CD4 receptors and certain coreceptors, are potential targets (e.g. dendritic cells, microglial cells, or glomerular epithelial cell)[33, 34]. The viral membrane protein gp120

binds to the coreceptors (also termed chemokine receptors), CCR5 or CXCR4, on the host cell and triggers conformational changes.

Next, the fusion takes place and the viral core is released into the cytoplasm. In the cytoplasm, the viral reverse transcriptase converts the HIV RNA into double-stranded HIV DNA. During this step, if a cell has been co-infected by two or more different viral HIV-1 strains, recombination can occur. In a next step, the HIV DNA is integrated into the host's chromosomal DNA. From now on, the host cell machinery for making cellular proteins is also used for making viral proteins. The integrated DNA provirus is transcribed into messenger RNA and exported from the nucleus into the cytoplasm where translation takes place. Afterwards budding from the cell membrane happens and the viral protease cleaves the large polyproteins to functional units. A mature virus is produced that is able to infect new host cells (reviewed in[35-37]).

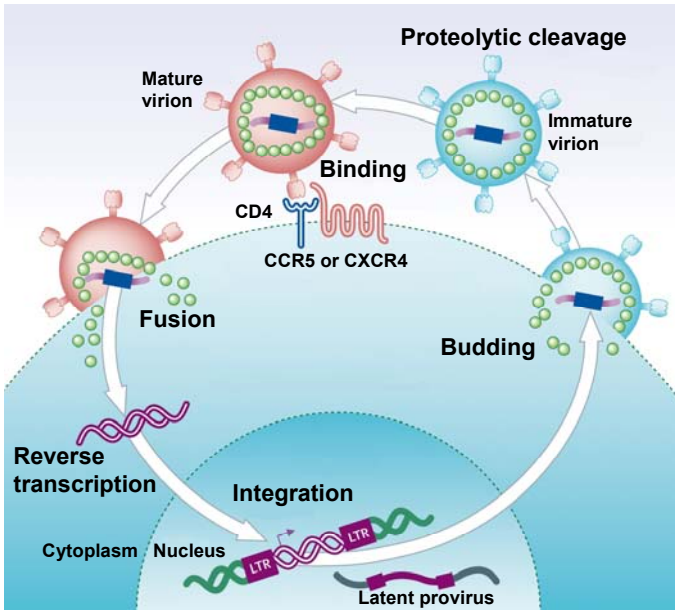

Figure 4. HIV-1 life cycle. Adapted from [37].

1.4 The natural history of HIV infections

The natural history of the HIV infection is characterized by three clinical stages: the acute, chronic and AIDS-defining phase (figure 5).[38] Within the first weeks after transmission, the level of HIV RNA often increases to very high levels and CD4+ cell count usually drop considerably.[39] In about 40-90% of newly infected patients, an acute retroviral syndrome (ARS) occurs. The ARS consists of variable symptoms such as fever, fatigue, pharyngitis, rash, headache, lymphadenopathy, diarrhea, myalgia or arthralgia. It is often clinically not distinguishable from other acute viral

syndromes (e.g. acute EBV infection and acute cytomegalovirus infection) and traveller's diseases (e.g. malaria, dengue virus infection, and travel-associated diarrhea). Thus, ARS remains often undiagnosed. [40-42]

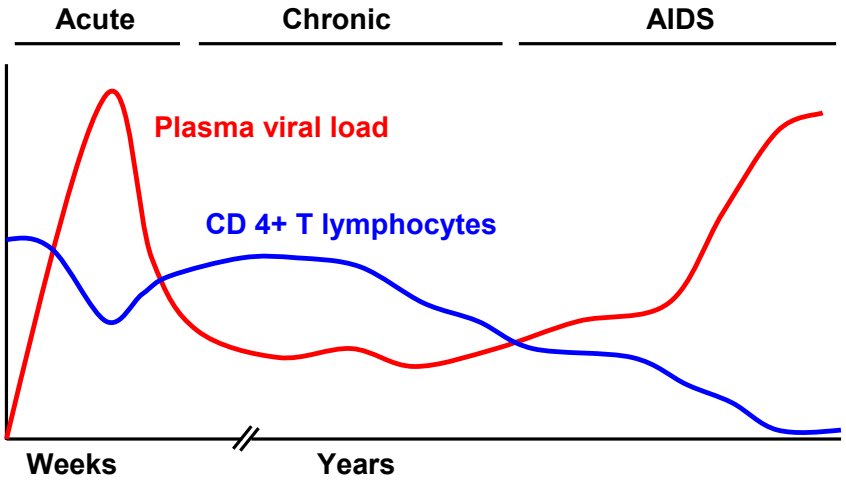

Figure 5. Natural course of an HIV-1 infection.

During the chronic phase of HIV infection, plasma viral load stabilizes at an individual level, the so-called viral set-point, and CD4+ cells often normalize to a certain extent. However, interpatient differences are extensive. A high viral set-point is associated with a more rapid loss of CD4+ cells and a faster progression to AIDS.[43, 44] However, the chronic phase can last several years and it is in most infected people asymptomatic. Untreated HIV-1 infected individuals will develop AIDS symptoms at a median of 8 to 10 years.[45] During the further course of infection a steady decrease of CD4+ cells occurs and the risk to enter the third stage, the AIDS-defining phase, increases with the decrease of the CD4+ cell count (reviewed in[35]). The AIDS-defining phase is symptomatic and defined by the occurrence of specific opportunistic infections (viral, bacterial, fungal, or parasitc diseases) and malign diseases. The Centers of Disease Control and Prevention (CDC) classified the phase of infection in so-called CDC stages depending on the disease progression.[38] In untreated patients, the average time between the first manifestations of AIDS and death is usually 2-4 years.[45]

1.5 Antiretroviral treatment

With the current antiretroviral therapy (ART) a cure from HIV is not possible. Therefore, the major aim of ART is to reduce the morbidity and mortality by fully

inhibiting active viral replication thereby suppressing the viral load. But HIV can survive in resting, latently infected cells, mostly of the CD4RO+ memory type, and it starts to replicate rapidly after ART is discontinued.[46-49] This rapid rebound suggests that viral replication persists at a very low level despite therapy and/or rapid reactivation of latently infected cells occurs when treatment is interrupted.[50-52] However, sustained viral suppression subsequently leads to a recovery of the CD4+ cell count in the large majority of patients. Antiretroviral compounds are usually classified by the viral life cycle step they inhibit (information about the mode of actions is given in sections 1.6.5-1.6.10). Currently, five major drug classes are approved in Switzerland including more than 20 different compounds. As shown in table 1, the first registered antiretroviral drug was zidovudine, a nucleoside reverse transcriptase inhibitor (NRTI).[53-55] However, mono- or dual-therapy with NRTIs was only transiently effective, because rapid selection of resistance occurred.[56, 57] The introduction of protease inhibitors (PIs) in 1995 was initially the major breakthrough in HIV treatment. The combination of a PI and NRTIs were proof of concept that triple therapy really made the difference and showed highly promising results in clinical trials. It was the first time that long-lasting viral suppression was achieved.[58] The term combination ART (cART) or highly active antiretroviral treatment (HAART) was introduced for the simultaneous administration of different drug classes. Today all PIs, except nelfinavir, if still used, and rarely atazanavir, are administered as ritonavir-boosted PIs.[59] Ritonavir is a very potent inhibitor of the hepatic enzyme cytochrome P450 (CYP) 3A4 that normally metabolizes PIs. The inhibition of this enzyme leads to a slower decrease of the PI drug levels. Therefore, a lower dosage, respectively longer dosing intervals are possible which reduces toxicity and pill burden immensely.[60] NNRTIs are an additional drug class approved in 1996.[61] Several years later, a fusion inhibitor (enfuvirtide) was registered.[62, 63] It is administered as a subcutaneous injection and adverse events are common, but it was until the registration of the CCR5 antagonists maraviroc and the integrase inhibitor (INI) raltegravir the only options for patient with multi-drug resistant viruses.[64-67]

To summarize, nowadays antiretroviral therapy is very potent and typically quite well tolerated. With a daily regimen of different drug classes long-term viral suppression can be achieved. The first-line ART usually consists of two NRTIs and a PI or a NNRTI, whereas drug combinations after multiple therapy failures are more complex

and usually include newer drug classes. Overall, the highly potent treatment, the reduction of pill burden and improved toxicity profiles changed HIV into a chronic illness with an estimated life expectancy comparable to other chronic diseases such as diabetes. [68-70] At least if treatment is available and affordable.

Table 1. Antiretroviral drugs against HIV-1 in Switzerland.

	Date of registration	Drug	Abbreviation	Brand name
NRTIs	1987	Zidovudine	AZT	Retrovir
	1992	Didanosine	DDI	Videx
	1992	Zalcitabine	DDC	Hivid
	1996*	Stavudine	D4T	Zerit
	1996	Lamivudine	3TC	Zeffix
	1999	Abacavir	ABC	Ziagen
	2002	Tenofovir	TDF	Viread
	2004	Emtricitabine	FTC	Emtriva
NNRTIs	1997	Nevirapine	NVP	Viramune
	1998	Efavirenz	EFV	Stocrin
	2008	Etravirine	ETV	Intelence
PIs	1996	Indinavir	IDV	Crixivan
	1996	Ritonavir	RTV	Norvir
	1997	Nelfinavir	NFV	Viracept
	1998	Saquinavir	SQV	Fortovase/Invirase
	1999*	Amprenavir	APV	Agenerase
	2000	Lopinavir	LPV	Kaletra
	2004	Atazanvir	ATV	Reyataz
	2005	Fosamprenavir	FPV	Telzir/Lexiva
	2005	Tipranavir	TPV	Aptivus
	2006	Darunavir	DRV	Prezista
Entry inhibitors	2003	Enfuvirtide	T20	Fuzeon
	2008	Maraviroc	MVC	Celsentri
Integrase inhibitor	2008	Raltegravir	RAL	Isentress

*Registration is expired. NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor

1.6 HIV drug resistance

HIV drug resistance is an important cause for treatment failure, although the introduction of cART reduced the emergence of drug resistance markedly in developed countries.[71-73] The simultaneous administration of different drug classes makes the development of resistant strains more difficult because it suppresses the viral replication more effectively and several mutations have to emerge on the same viral genome until viruses are able to replicate.[74] However, if intracellular drug levels decrease, e.g. because of drug-drug interactions or missed doses, latently infected cells may be reactivated and the replication cycle restarts. A Darwinian selection process takes place and the viral strain with the highest replication capacity, or viral fitness, takes over the viral population. The selection of mutations is a possible

adaption of the viral strain to increase its replication capacity under drug pressure. We are talking about drug resistant strains, if viruses are able to replicate besides appropriate drug levels (reviewed in [74, 75]).

There are two main reasons why HIV drug resistance can occur quite rapidly. First, HIV has a very high mutation rate. The reverse transcriptase of HIV lacks a proofreading activity. It is not able to confirm that the DNA transcript is an accurate copy of the RNA. It introduces on average one mutation for each viral genome transcribed.[76, 77] The second reason is the high replication rate of HIV.[78] In untreated individuals, it is estimated that 10^7 to 10^8 cells are infected.[79] They have an average life time of 1-2 days.[80, 81] It is obvious that the replication rate has to be very high to maintain a steady state. Thus, the combination of the error prone reverse transcriptase and the high replication rate leads to the generation of each possible mutation in an HIV-1 individual every day.[82] Further, recombination of different viral strains can occur during replication which increases the viral diversity even more (see section 1.3).

1.6.1 Nomenclature of mutations associated with drug resistance

Proteins are built of a chain of amino acids. Twenty different amino acids exist and their sequence in a protein is defined on the genome. An amino acid is encoded by a three-letter code of nucleotides (codon). Mutations emerge when this three-letter code is modified by replacement, deletion or insertion of nucleotides. Mutations can be synonymous or non-synonymous. A synonymous mutation occurs when a change of a nucleotide does not result in the expression of another amino acid. This is possible because there are 4^3=64 codons, but only 20 amino acids. The change at the third position is quite often synonymous or silent, e.g. lysine is encoded by AAA and AAG. Relevant drug resistance-associated mutations are non-synonymous, meaning that the change leads to an expression of another amino acid, e.g. when the code of lysine (AAA) is modified at the second position to AGA, arginine is expressed.

The nomenclature of drug resistant-associated mutations has two letters and a number. The first letter is the abbreviation for the amino acid of the wild type (consensus strain), the number is the position of the mutation on the genome, and the second number is the abbreviation of the amino acid of the mutant strain. The mutation K103N for example is a substitution of the amino acid lysine (K) with asparagine (N) at position 103.[83]

1.6.2 Impact of mutations associated with drug resistance on viral fitness

Viral strains often have a decreased viral fitness in the presence of drug resistance mutations, M184V for example has a large impact on the replication capacity.[84] But other mutations, so-called secondary or accessory mutations, can be selected that partially compensate for this effect. However, if ART is cessed, the wild type strain most often has a better replication capacity and therefore, drug resistant strains may partially or fully diminish to undetectable levels during treatment interruptions. This is a reason why genotypic resistance tests must be interpreted with care when performed before ART initiation or during treatment interruptions.[83, 85]

1.6.3 HIV drug resistance testing

The major indicator for the presence, respectively for the emergence of drug resistance is a relapse of a detectable viral load in a previously suppressed patient on ART or lack of initial treatment response. Generally, HIV drug resistance is measured with two assays, a genotypic and phenotypic test. These assays have different assets and drawbacks.[83] Both tests suffer from the limitation that a minimum amount of virus (>500 copies/mL) is necessary to perform the test and minority subpopulations can not be detected. Viral strains that represent <20% of the total population may remain undetected with these assays.[86, 87]

Genotypic resistance test

The genotypic resistance test predicts the susceptibility of antiretroviral drugs based on the viral sequence. To estimate the activity of PIs, NRTIs, NNRTIs and INIs parts of the *pol* region are sequenced. To predict the susceptibility to the fusion inhibitor enfuvirtide, a part of the *env* region is sequenced.

The major limitation of the genotypic resistance tests is the interpretation of the results, because the impact of different mutations on drug susceptibility varies widely. Therefore, several interpretation algorithms were developed to estimate the activity of ART based on the viral sequence. Some of the widely used algorithms are the Stanford [http://hivdb.stanford.edu/pages/algs/HIValg.html], the REGA[88] or ANRS algorithms [www.hivfrenchresistance.org/index.html]. Additionally, the international aids society (IAS-USA) regularly publishes recommendations on how to interpret resistance mutations.[89] However, the concordance of the algorithms is not always given which can make the interpretation of resistance tests difficult.[90, 91]

Phenotypic resistance test

Phenotypic resistance tests are direct *in vitro* measurements to determine the sensitivity of viruses to particular drugs. Originally, replication competent viral isolates from patients were grown in presence and absence of drugs in cell culture. However, for better standardization recombinant assays have been developed. For this purpose, a recombinant virus is constructed inserting protease and reverse transcriptase genes of patient's viruses into a HIV reference strain. These recombinant viruses are than tested *in vitro* for the amount of drugs needed to inhibit the viral replication by 50%. This level is called the half maximum inhibitory concentration (IC_{50}). For interpretation, a clinical cut-off indicates at which levels of IC_{50} a successful treatment can still be expected. However, the determination of these cut-offs is difficult.[92]

In contrast to genotypic resistance test, the phenotypic resistance is rarely used in clinical practice. It is more time consuming and more expensive than the genotypic test. Currently, the most commonly used commercial phenotypic tests are PhenoSense [http://www.monogrambio.com/417.aspx] and Virco's Antivirogram [http://www.vircolab.com/hiv-resistance-products/antivirogram].[93]

Virtual phenotype

The virtual phenotype is a mathematical approach to predict the phenotype based on the genotypic information without performing a phenotypic resistance test in laboratory. These estimates are derived from large databases that combine information from phenotypic and genotypic tests. There are two widely used interpretation system, geno2pheno [http://www.geno2pheno.org/] and vircoType [http://www.vircolab.com/hiv-resistance-products/vircotype-hiv-1]. Geno2Pheno uses machine learning techniques[94, 95] to determine the phenotype, whereas vircoType interpretation is based on multiple, linear regression models.[96]

Minority variants harbouring drug resistance

Ultrasensitive methods, such as allele-specific real-time PCR and deep sequencing, were developed to achieve higher sensitivities for genotypic resistance tests. These assays are able to detect viral strains occurring at very low frequency (up to 0.1%), but are only used for research purpose and not in routine clinical care yet.[87, 97-99] They are labour intensive and are not approved for diagnostic testing.

However, the clinical relevance of low-frequency mutations is still under investigation.[100-102] But a recently published systematic review found evidence that the presence of low-frequency mutations, in particular NNRTI mutations, is associated with an increased risk for virological failure.[103]

1.6.4 Transmission of HIV drug resistance

In Western countries, the prevalence of transmitted drug resistance is about 8-15%.[26, 104-109] Less data are available from resource-limited settings, but recent studies reported comparable rates.[110-114] However, a correlation was found between the prevalence of transmitted drug resistance and the time since ART scale-up in these countries. Therefore, it is possible that rates are increasing in the next few years.[115] Transmission of minority variants harbouring drug resistance mutations also occurs, but the clinical relevance is currently unknown.[116]

In Switzerland, the transmission rate of HIV drug resistance remained stable between 1996 and 2005 and was approximately 8%.[26] The transmission rate may be associated with the prevalence of drug resistance in the population which reached a plateau in the last few years, or is even decreasing in Switzerland.[71]

The presence of transmitted drug resistance is associated with a worse treatment outcome.[117-121] It is recommended to perform a genotypic resistance test prior to first-line ART and to adapt the treatment to the drug resistance profile.[122]

1.6.5 NRTI resistance-associated mutations

NRTIs are nucleoside/tide analogues. They compete with natural nucleotides to be incorporated in the viral genome during the reverse transcription. In contrast to natural nucleotides, NRTIs lack a 3' hydroxyl group and after its incorporation the reverse transcription is interrupted, because no further nucleotides can be attached.

However, mutations exist that affect the efficacy of NRTIs. In general, NRTI mutations act in two different ways, they reduce the ability to incorporate NRTIs in the genome during reverse transcription (e.g. K65R, L74V, M184V or the Q151M complex) or lead to an ATP dependent removal of NRTIs after its incorporation (e.g. thymidine analogue mutations [TAMs]) (reviewed in[74, 75]). Depending on the type of selected mutation, different levels of resistance against drugs emerge (figure 6).

Cross-resistance within drug classes is often problematic, also among NRTIs. Cross-resistance occurs if a mutation is selected by a specific drug and the presence of this mutation additionally leads to resistance against other drugs. K65R for example is

most often selected by tenofovir, but if K65R is present, the viral strain is resistant against several other NRTIs.

The reverse phenomenon also occurs. A mutation selected by a particular drug can lead to hyper-susceptibility to another drug. An example is M184V. It is a very common NRTI mutation. The presence of M184V results in high level resistance to lamivudine and emtricitabine, but increases the susceptibility to zidovudine, stavudine and tenofovir, most likely by reducing ATP-dependent nucleotide excision.[123, 124] M184V has a low genetic barrier meaning that selection of drug resistance occurs rapidely. The genetic barrier is defined as the number of viral changes needed to overcome the drug-selective pressure.[125]

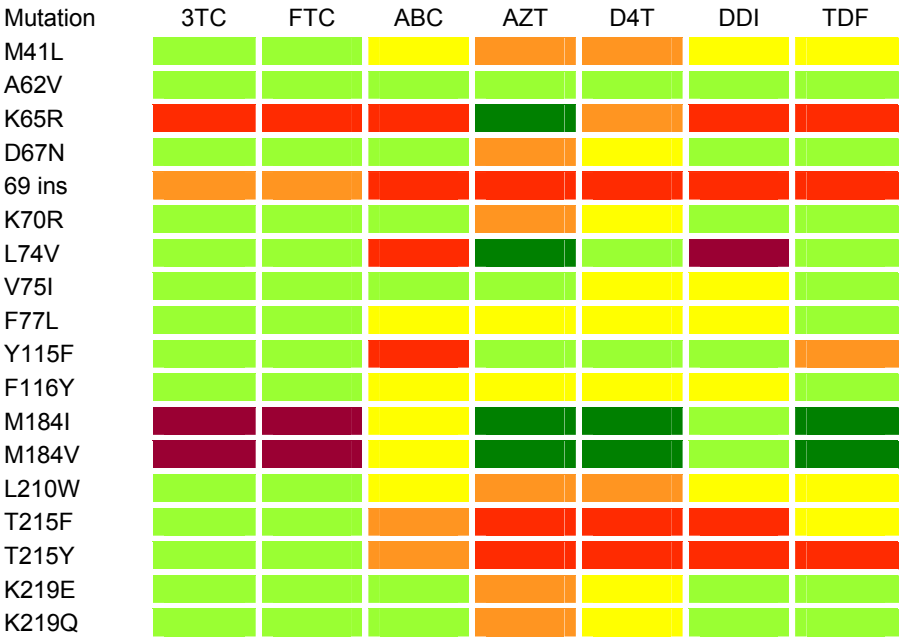

Figure 6. Estimated susceptibility of nucleoside reverse transcriptase inhibitors in the presence of particular mutations. Susceptibility is estimated based on Stanford algorithm (version 6.0.11). Colour code: ■ hypersuceptible, ■ susceptible, ■ potential low-level resistance ■ low-level resistance, ■ intermediate resistance, ■ high-level resistance. 3TC, lamivudine; FTC, emtricitabine; ABC, abacavir; AZT, zidovudine; D4T, stavudine; DDI, didanosine; TDF, tenofovir.

The type of mutation that emerges is highly dependent on the drugs used, but also interaction between mutations play a role. Mutations can occur as clusters, e.g. TAMs emerge as two different patterns. The TAM 1 pattern includes M41L, L210W, and T215Y and the TAM 2 pattern consists of D67N, K70R, T215F, and K219Q/E, but the two patterns are not fully exclusive.[126-130] The interaction between mutations can also be an important criterion to choose a drug combination. For example, M184V acts antagonistically with TAMs. The use of thymidine analogue (zidovudine

or stavudine) with lamivudine or emtricitabine results in lower resistance rates.[131] In addition, combination of thymidine analogue and tenofovir, basically precludes the emergence of tenofovir resistance, even if the virus is harbouring resistance mutation against thymidine analogue.[132]

1.6.6 NNRTI resistance-associated mutations

In contrast to NRTIs, NNRTIs are not incorporated in the viral genome during reverse transcription. NNRTIs are small molecules that bind to a hydrophobic pocket close to the catalytic site of the reverse transcriptase. Their binding causes a conformational change in the reverse transcriptase resulting in an inhibition of the polymerization. NNRTI mutations are almost all located in the hydrophobic pocket or nearby. They decrease the binding affinity of the drug.[133-135]

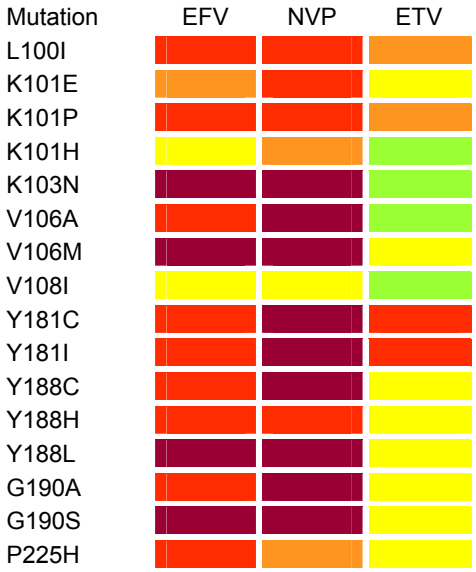

Figure 7. Estimated susceptibility of nonnucleoside reverse transcriptase inhibitors in the presence of particular mutations. Susceptibility is estimated based on Stanford algorithm (version 6.0.11). Colour code: ■ hypersuceptible, ■ susceptible, ■ potential low-level resistance ■ low-level resistance, ■ intermediate resistance, ■ high-level resistance). EFV, efavirenz; NVP, nevirapine; ETV, etravirine

NNRTIs generally have a low genetic barrier, only one or two mutations are needed for high-level resistance (e.g. K103N).[136] The selection of particular mutations is usually dependent on the drugs used. Efavirenz selects mostly for K103N, but also for Y188L and Y181C. Nevirapine selects more often for Y181C, but also other mutations such as K103N, V106, Y188C and A190A occur. As shown in figure 7, cross-resistance is an important problem among NNRTI. If a viral strain is resistant against efavirenz, it is usually also resistant against nevirapine and vice versa. In

2008, a new generation NNRTI, etravirine, entered the market. It has a different resistance pattern and cross-resistance is clearly reduced. Especially the common mutation, K103N, has no or only little impact on etravirine susceptibility.[137, 138]

TAMs are associated with a hyper-susceptibility to NNRTIs, although the clinical significance of hyper-susceptibility is currently not known.[139, 140]

1.6.7 PI resistance-associated mutations

The HIV protease cleaves large precursor polyproteins and generates functional subunits. If this action is inhibited viral particles are still produced, but they are noninfectious. Most PIs have been designed to mimic the natural substrates of the viral protease and they bind with a high affinity to the active site of the protease (reviewed in[74, 75]). This process hampers the cleavage of polyproteins. PI mutations are usually located inside the substrate-binding domain of the protease or at neighboring sites. PI mutations are classified in major and minor mutations. Major PI mutations have the largest clinical impact on susceptibility and occur at 13 positions on the protease. Minor PI mutations, also called accessory or secondary mutations, either improve the viral fitness or increase drug resistance.[141] Minor mutations do not

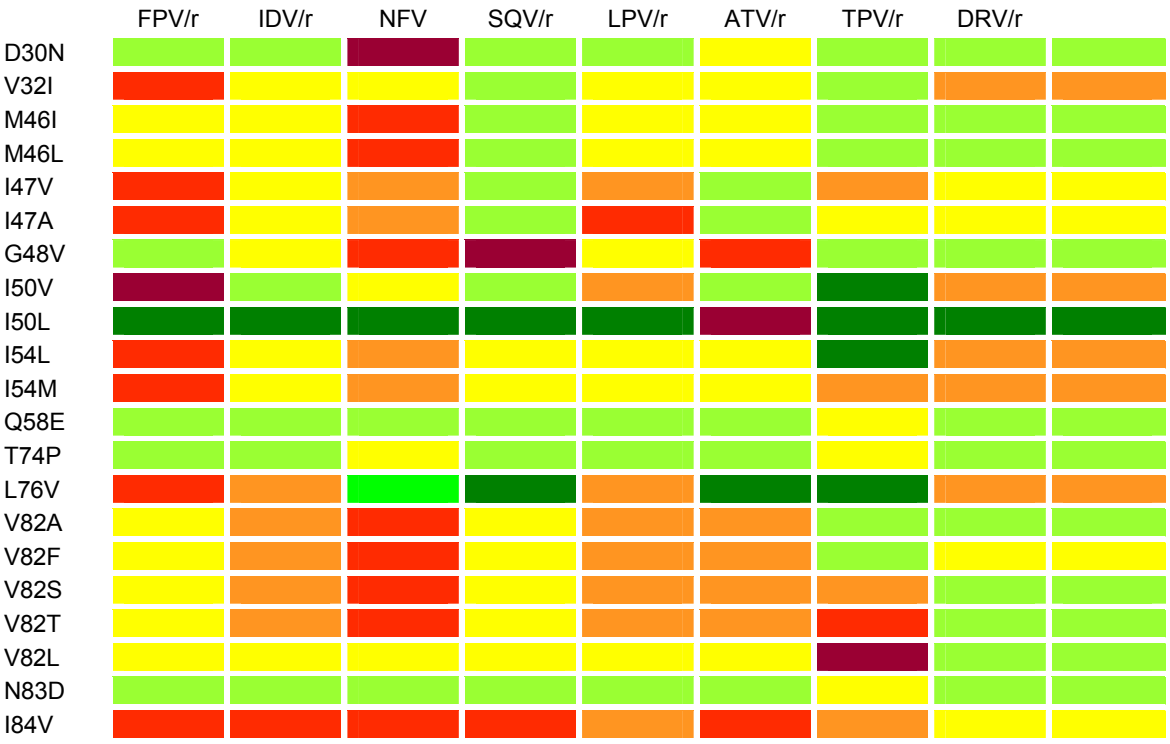

Figure 8. Estimated susceptibility of protease inhibitors in the presence of particular mutations. Susceptibility is estimated based on Stanford algorithm (version 6.0.11). Colour code: ■ hypersuceptible, ■ susceptible, ■ potential low-level resistance ■ low-level resistance, ■ intermediate resistance, ■ high-level resistance. FPV, fosamprenavir; IDV, indinavir; NFV, nelfinavir; SQV, saquinavir; LPV, lopinavir; ATV, atazanavir; TPV, tipranovir; DRV, darunavir; /r, ritonavir boosted

lead to high-level resistance when occurring alone, but evidence was found that the time to virological failure is shortened when specific minor mutations are present.[142, 143] Minor PI mutations occur quite frequently as polymorphisms and its prevalence differs largely between subtypes.[144]

Cross-resistance is also an important issue among PIs (figure 8). But the genetic barrier to develop resistance is higher for PIs compared to NNRTIs or some NRTIs.

1.6.8 Fusion inhibitor resistance-associated mutations

When HIV enters target cells gp41 interacts with chemokine receptors on the cell surface and during the entry process the two hydrophobic regions HR1 and HR2 bind to enable HIV's entry. Enfuvirtide, a small peptide, the only registered fusion inhibitor, destabilizes this process. It mimics a part of the HR2 and binds to a conserved part of HR1.[145] Enfuvirtide is only used in salvage therapy and in absence of other active or partially active drugs resistance mutations occur rapidly.[146] Drug resistant mutations emerge at position 36 to 45 in the gp41. This region is part of the HR1 where T20 is binding.[147-149] A single mutation usually leads to a 10-fold decrease in susceptibility and the occurrence of a second mutations leads up to a 100-fold reduction in susceptibility.[150] There are several accessory mutations in the HR2 region which can compensate for the loss of viral fitness.

1.6.9 CCR5 inhibitor resistance-associated mutations

CCR5 inhibitors affect gp120 binding to the CCR5 co-receptor by an allosteric binding mechanism. Maraviroc is currently the only registered CCR5 inhibitor.[64, 65] It is only effective when viruses exclusively use CCR5 for entry and not CXCR4. In the early stage of an HIV infection about 80-99% of the patients have viruses that exclusively use CCR5, but in later stages, the percentage of patients using CXCR4 increases.[151, 152] Different assays exist to determine the coreceptor usage (e.g. Trofile, a single-cycle recombinant virus assay in which a pseudovirus is produced from the full length *env* genes from patient's virus population), but also genotypic prediction algorithms exist where the phenotypic activity is estimated based on genotypic information mostly of the V3 region, such as geno2pheno [http://coreceptor.bioinf.mpi-inf.mpg.de/index.php], Web PSSM [http://indra.mullins.microbiol.washington.edu/webpssm/], or Wetcat [http://genomiac2.ucsd.edu:8080/wetcat/v3.html]. However, rarely drug resistance can also emerge independent of the tropism. It was shown that some mutations located in the V3 loop of gp120 are

associated with maraviroc resistance but there is no consensus about the clinical relevance of these mutations.[89, 153, 154]

1.6.10 INI resistance-associated mutations

The HIV integrase incorporates the double-stranded cDNA in the host's genome. This process includes generally three steps: 1) formation of the preintegration viral DNA complex, 2) 3' processing, and 3) strand transfer. INIs hamper the last step.[155]
As in other drug classes, primary and secondary mutations occur during treatment with INIs. The selection of secondary mutations leads to further loss of activity or improved viral fitness. The single occurrence of mutation Y143R, Q148H/K/R or N155H is sufficient to lead to considerable reduction of INI activity. The genetic barrier to INI resistance is rather low.[66, 67]

1.7 The Swiss epidemic and the Swiss HIV Cohort Study

In contrast to Sub-Saharan Africa where HIV transmission mostly occurs during heterosexual contacts, HIV transmission in Switzerland currently occurs most often by homosexual intercourse between men (about 50%) [http://www.bag.admin.ch/hiv_aids/]. The number of infections caused by intravenous drug use decreased in the last decade markedly, most likely due to the free delivery of needles, methadone and heroine programs. Furthermore, the role of intravenous drug users in transmission to the heterosexual population diminished over time.[156] It is estimated that about 19,000-26,000 HIV infected patients live in Switzerland. Between 2000 and 2010, the number of newly diagnosed persons was usually between 600 and 800 per year. This results in a rate of 8.0-10.9 cases per 100,000 people [http://www.bag.admin.ch/hiv_aids/]. The number of AIDS-related death decreased markedly since the introduction of cART in 1995.[157]
The Swiss HIV Cohort Study (SHCS) was established in 1988. It is an observational, multi-centre study of HIV infected individuals in Switzerland. The study is a collaboration of seven centres: Basel, Bern, Geneva, Lausanne, Lugano, St. Gallen, and Zürich. The SHCS has been approved by ethical committees of all participating institutions and written informed consent has been obtained from all participants. Overall the SHCS is estimated to include about 69% of patients living with AIDS and approximately 45% of patients infected with HIV in Switzerland.[158] Until December 31, 2010, 16,778 individuals were registered (figure 9). At registration, basic information is collected (e.g. year of birth, sex, transmission category and ethnicity)

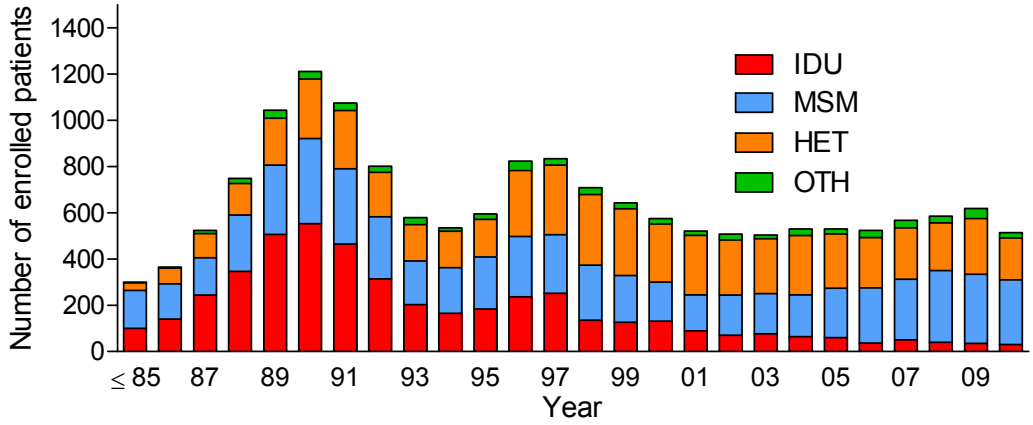

Figure 9. Patients enrolled in the SHCS stratified by the transmission category. Adapted from www.shcs.ch. IDU, intravenous drug user; MSM, men who have sex with men; HET, heterosexual; OTH, other.

and further on, laboratory and clinical data are collected at each semi-annual study visit. Also additional performed laboratory measurements, such as CD4+ cell count or viral load, are stored. Further, the antiretroviral treatment is recorded in detail, but also adherence data and adverse events are available.

1.8 The SHCS drug resistance database

Since 2000, genotypic resistance tests are performed in routine clinical care. In Switzerland, four laboratories are authorised from the Swiss Federal Office for Public Health to perform these tests. All laboratories use population-sequencing methods. The full protease gene and in minimum codon 28 to 225 of the reverse transcriptase gene are sequenced using commercial assays (Viroseq Vs. 1 PE Biosystems, Rotkreuz, Switzerland; Virsoseq Vs. 2, Abbott AG, Baar, Switzerland; vircoTYPE HIV-1 Assay, Virco Lab, Mechelen, Belgium) and in-house methods.[159] In 2001, it was decided to store the sequences in a central database (SmartGene's Integrated Database Network System, Zug, Switzerland) and to link the sequences to the SHCS with the SHCS identification number. In the first years, problems occurred with the data transfer of SHCS identification numbers to the laboratories and the linkage remained incomplete. In 2005, a large effort was undertaken to link data to the SHCS and in the following years, several thousand resistance test were performed retrospectively from stored plasma samples to complete the data set (von Wyl, Günthard, Scherrer, unpublished data).

At the end of 2010, 16,399 sequences were stored in the database (figure 10). Out of these, 13,980 sequences from 9,660 different patients were linked to the SHCS.

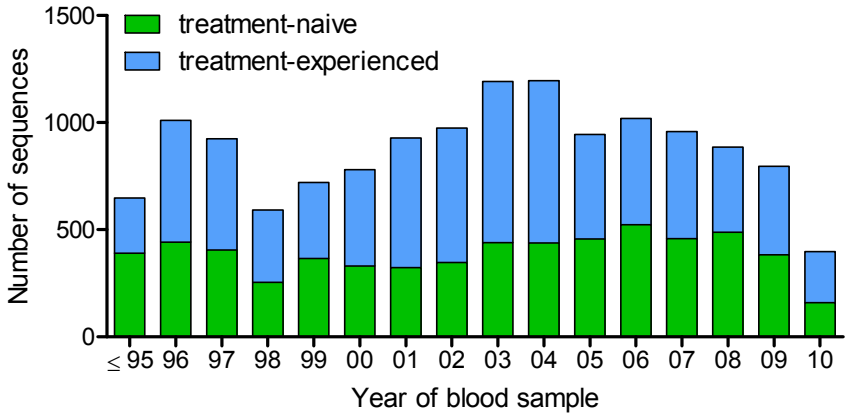

Figure 10. Number of sequences stored in the SHCS drug resistance database stratified by samples from treatment-naïve and treatment-experienced patients.

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Chapter 1

Estimate of etravirine activity

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Prevalence of etravirine mutations and impact on response to treatment in routine clinical care: the Swiss HIV Cohort Study (SHCS)

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Abstract

Objectives

Etravirine (ETV) is a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) with reduced cross-resistance to first generation NNRTIs, which has been primarily studied in randomized clinical trials and not in routine clinical settings.

Methods

ETV resistance-associated mutations (RAMs) were investigated by analysing 6072 genotypic tests. The antiviral activity of ETV was predicted using different interpretation systems: International AIDS Society-USA (IAS-USA), Stanford, Rega and Agence Nationale de Recherches sur le Sida et les hépatites virales (ANRS).

Results

Prevalence of ETV RAMs was higher in NNRTI-exposed [44.9%, 95% confidence interval (CI) 41.0-48.9%] than in treatment-naïve patients (9.6%, 95% CI 8.5-10.7%). ETV RAMs in treatment-naïve patients with documented recent (<1 year) infection, who had acquired HIV before the introduction of NNRTIs, were almost identical (9.8%, 95% CI 3.3-21.4). Discontinuation of NNRTI treatment led to a marked drop in the detection of ETV RAMs, from 51.7% (95% CI 40.8-62.6%) to 34.5% (95% CI 24.6-45.4%, P=0.032). Differences in prevalence among subtypes were found for V90I and V179T (P<0.001). Estimates of restricted virological response to ETV varied among algorithms in patients with exposure to efavirenz (EFV)/nevirapine (NVP), ranging from 3.8% (95% CI 2.5-5.6%) for ANRS to 56.2% (95% CI 52.2-60.1%) for Stanford. The predicted activity of ETV decreased as the sensitivity of potential optimized background regimens decreased. The presence of major IAS-USA mutations (L100I, K101E/H/P, Y181C/I/V) reduced the treatment response at week 24.

Conclusions

Most ETV RAMs in drug-naïve patients are polymorphisms rather than transmitted RAMs. Uncertainty regarding predictions of antiviral activity for ETV in NNRTI-treated patients remains high. The lowest activity was predicted for patients harbouring extensive multidrug-resistant viruses, thus limiting ETV use in those who are most in need.

Introduction

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are important components in the drug combination schemes that are currently used in the treatment of HIV-1 infections.[1, 2] Because of overlapping resistance profiles of currently approved NNRTI drugs, cross-resistance can emerge rapidly, which excludes future use of this drug class.[3-5] Recently, the situation has changed with the introduction of the diarylpyrimidine derivative etravirine (ETV), a novel, next generation NNRTI.[6] ETV was designed for activity against wild-type HIV-1 and against strains harbouring NNRTI resistance-inducing mutations selected by nevirapine (NVP) or efavirenz (EFV). Its resistance-associated mutation (RAM) pattern is partially different from that of other NNRTIs and its genetic barrier, defined as the number of mutations required to confer full resistance, seems to be considerably higher.[7, 8] Clinical studies showed that the presence of three or more ETV RAMs led to substantially decreased virological response.[9-12] Current International AIDS Society-USA (IAS-USA) recommendations list the following 14 ETV RAMs: V90I, A98G, L100I, K101E/P, V106I, V179D/F/T, Y181C/I/V and G190A/S.[13, 14] Recently, Vingerhoets et al.[11] have updated the list of mutations and added K101H, E138A and M230L. Of note, this list does not include K103N, which confers high-level resistance to both EFV and NVP.

The potential eligibility of patients for treatment with ETV in routine clinical practice has not been systematically assessed for large, well-characterized drug-naïve and treatment-experienced individuals. For this reason, we interrogated the resistance database from the Swiss HIV Cohort Study (SHCS) to determine the prevalence of ETV RAMs and to predict the potential eligibility of treatment-naïve and treatment-experienced patients for treatment with ETV. Moreover, we compared different algorithms that predict susceptibility to ETV based on available genotypic resistance tests (GRTs). As it has been shown repeatedly that new antiretroviral drugs should be accompanied by at least two additional active compounds for optimal virological response to salvage treatment,[15-19] we further identified patients who had failed NNRTIs previously with at least two remaining drugs for the background regimen in addition to ETV.

Methods

Data and patient selection

Our analysis included clinical and genotypic data collected up to May 2008. The SHCS is a nationwide, clinic-based cohort study with continuous enrolment and at least bi-annual study visits.[20] The SHCS has been approved by ethical committees of all participating institutions and written informed consent has been obtained from participants. The SHCS resistance database contains all genotypic HIV resistance tests performed by the four authorized laboratories in Switzerland, stored in SmartGene's (Zug, Switzerland) Integrated Database Network System (IDNS version 3.4.0).[21] Resistance tests performed between January 1999 and May 2008 were included in this study. Patients with recent infection were defined as presenting with either documented acute infection or a documented sero-conversion within 1 year as described in detail elsewhere.[22]

Analysis

Because GRTs were obtained under various circumstances, we stratified our analysis by whether patients were treatment naïve (group A) or antiretroviral therapy (ART) exposed at the time of resistance testing. Samples from treatment-experienced patients were grouped further according to NNRTI exposure. Group B included resistance tests performed in treatment-experienced patients never exposed to any NNRTI, group C included resistance tests performed while patients were receiving NNRTI treatment and group D included tests performed after exposure to NNRTI (58.4% on treatment without NNRTIs, 41.6% off treatment). We only considered tests that were performed after at least 30 days of continuous exposure to ART since the last treatment modification. Patients could appear in more than one group, but only the latest resistance test of a patient was included if several tests per patient were available for the same group.

Resistance mutations against ETV were defined according to the mutation list of the International AIDS Society-USA (IAS-USA).[13, 14] We further classified ETV RAMs into ETV-specific mutations, which do not show cross-resistance to EFV or NVP based on the IAS-USA recommendations, and nonspecific mutations, which also confer resistance to NVP and EFV. The ETV-specific mutations included V90I, A98G,

K101E/H/P, V106I, E138A, V179D/F/T, Y181V and M230L, and ETV nonspecific mutations consisted of L100I, Y181C/I and G190S/A.

GRTs were categorized into fully susceptible, intermediately resistant, and fully resistant to a specific antiretroviral drug with three frequently used interpretation algorithms: the Stanford algorithm, version 5.0.1,[23] the RegaV7 1.1 algorithm[24] and the Agence Nationale de recherches sur le sida et les hépatites virales (ANRS) (http://www.hivfrenchresistance.org/index.html) version 16 algorithm as implemented in the Stanford HIV Drug Resistance algorithm comparison tool (hivdb.stanford.edu/pages/algs/HIValg.html). As a fourth classification following the IAS-USA spring 2008 guidelines, we considered viruses with at least three IAS-USA ETV RAMs as fully resistant to ETV and viruses with one or two IAS-USA ETV RAMs as intermediately resistant. The newest update of IAS-USA guidelines, for December 2008, has assigned relative weights to the following mutations based on *in vitro* and in *vivo data*: L100I, K101E/H/P and Y181C/I/V.[13, 14] In analogy to major protease inhibitor (PI) mutations it has been shown that these weighted ETV mutations do induce a substantial reduction in phenotypic drug susceptibility compared with the other nonweighted ETV mutations.[25-27] Thus, for simplicity, we will call ETV RAMs at these three positions as 'major ETV mutations'. To assess the availability of active antiretroviral compounds for combination with ETV, results obtained using the Stanford interpretation algorithm were mapped to a genotypic sensitivity score (GSS) for all approved drugs except enfuvirtide, maraviroc and raltegravir. Drugs with a score of 0 were considered inactive because of full resistance of the virus to that compound, 0.5 indicated intermediate antiretroviral activity and 1 related to full activity based on GRTs. It was assumed that ETV-based salvage therapy following EFV or NVP failure should consist of the two highest scoring NRTI drugs and one of the highest scoring boosted PIs as background treatment. Thus a maximum score of 3 could be achieved for a fully active background regimen and a score of 0 if all drugs were considered inactive. Virological failure was defined as viral rebound after previous suppression with two consecutive viral loads >500 HIV-1 RNA copies/mL or a single value >500 copies/mL followed by a stop or a modification of the current therapy.

Statistical analysis was performed with Stata 10 SE (StataCorp, College Station, TX, USA). Proportions were compared with the Fisher's exact test. All confidence

intervals (CIs) were two-sided 95% confidence intervals and calculated with the Clopper-Pearson method. The level of significance was set at $P<0.05$.

Results

Prevalence of RAMs for ETV

We included 6072 resistance tests from the SHCS drug resistance database in our analyses: 3020 (49.7%) GRTs performed in treatment-naïve patients (group A), 1667 (27.5%) in NNRTI-naïve patients exposed to ART without NNRTIs (group B), 639 (10.5%) performed in patients on NNRTI treatment (group C) and 746 (12.3%) performed in patients with past NNRTI experience (group D) (Fig. 1).

ETV RAMs occurred three times more frequently in NNRTI-experienced patients (groups C and D: 32.6%, CI 30.2-35.2%) compared with NNRTI-naïve patients (groups A and B: 10.0%, CI 9.1-10.9%, $P<0.001$). In patients with virological failure on EFV/NVP the prevalence was 51.9% (CI 45.7-58.2%). The prevalence of nonspecific ETV RAMs, which also confer resistance to other approved NNRTIs, was higher in NNRTI- experienced patients (groups C and D: 21.4%, CI 19.2-23.6%) than in NNRTI-naïve patients (groups A and B: 0.7%, CI 0.5-1.0%, $P<0.001$). The increase in frequency of specific ETV RAMs following exposure to NNRTI was less pronounced, but still highly significant (groups A and B: 9.5%, CI 8.7-10.4%; groups C and D: 19.9%, CI 17.9-22.1%, $P<0.001$). Among patients with at least one ETV RAM, single mutations were most common (84.8%, CI 83.9-85.7%). The acquisition of three or more ETV RAMs, which would indicate high level resistance to ETV according to IAS-USA, was most common in groups C and D (8.2%, CI 5.8-11.1%) and occurred significantly less frequently in NNRTI-naïve patients (groups A and B: 0.9%, CI 0.2-2.2%, $P<0.001$). Also, the occurrence of at least one major ETV RAM according to IAS-USA (L100I, K101E/H/P, Y181C/I/V) was higher in groups C and D (18.8%, CI: 16.7-20.9%) compared with NNRTI-naïve patients (groups A and B: 0.9%, $P<0.001$).

As illustrated in Figure 2, the most prevalent mutations in NNRTI-naïve patients (groups A and B) were the ETV-specific mutations E138A (group A: 2.8%; group B: 2.8%), V106I (group A: 2.3%; group B: 1.7%) and V90I (group A: 2.1%; group B: 2.9%), whereas in patients tested while receiving NNRTIs (group C) or after NNRTI exposure (group D) mutations selected by EFV and NVP were mainly dominating: L100I (8.3%), Y181C (15.0%), G190A (12.5%) in group C and V90I

(3.9%), Y181C (5.0%) and G190A (3.2%) in group D. Interestingly, the two ETV RAMs V179F and Y181V were not found at all in our large study sample.

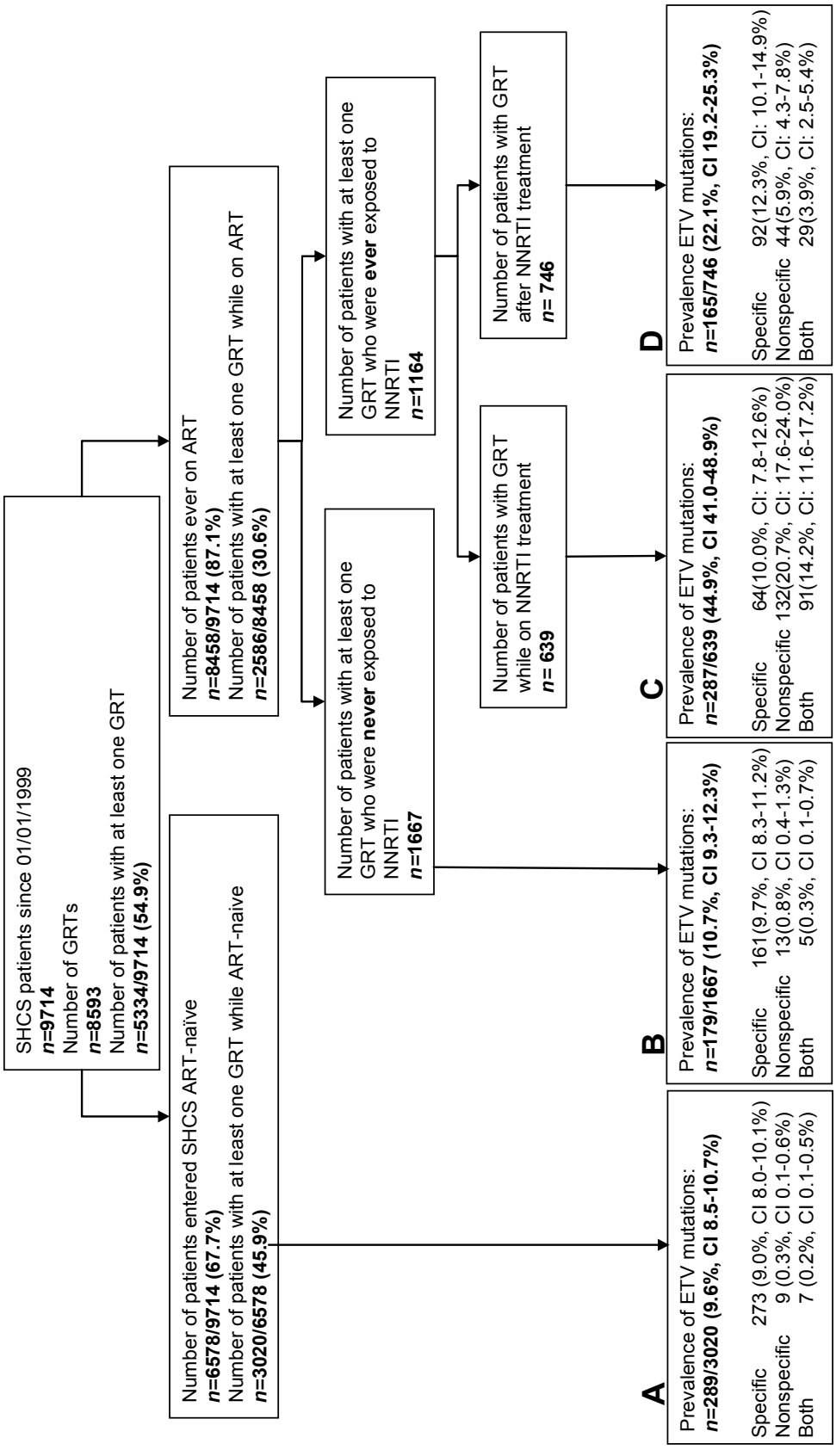

Figure 1. Study design and prevalence of etravirine resistance-associated mutations (ETV RAMs). The study population was subdivided into four groups depending on the circumstances of the genetic resistance test. (GRT) Group A: GRT performed in treatment-naïve patients; group B: GRT performed in treatment-experienced patients never exposed to any nonnucleoside reverse transcriptase inhibitor (NNRTI); group C: GRT performed in patients receiving NNRTIs; group D: GRT performed at least 30 days after exposure to NNRTIs. ETV RAMs were defined according to the mutation list of the International AIDS Society-USA (IAS) and subdivided into specific (mutations that affect ETV only) and nonspecific ETV RAMs [mutations that also confer resistance to nevirapine (NVP)/efivarenz (EFV)]. 95% confidence intervals (CI) are indicated. SHCS, Swiss HIV Cohort Study; ART, anti-retroviral therapy.

Association between ETV RAMs and HIV subtypes

We further analysed associations between ETV RAMs and HIV subtypes based on genotypic tests from treatment-naïve patients (group A, n=3020). Most samples were subtype B (78.1%), followed by subtype CRF02_AG (6.2%), A (5.5%), C (5.4%) and CRF01_AE (4.7%). Based on Fisher's exact test and Bonferroni-adjustment of *P*-values for multiple testing, two mutations were found to be significantly different between subtypes, V90I and V179T (both $P<0.001$). V90I was most prevalent in subtype CRF02_AG (7.9%, CI 4.3-13.2%) compared with subtypes A (2.1%, CI 0.4-5.9%) and B (1.9%, CI 1.4-2.6%) and did not occur in subtypes C. V179T was most common in subtypes A (4.1%, CI 1.5-8.7%) and CRF01_AE (3.2%, CI 0.9-8.1) and was not found in subtypes CRF02_AG, B and C.

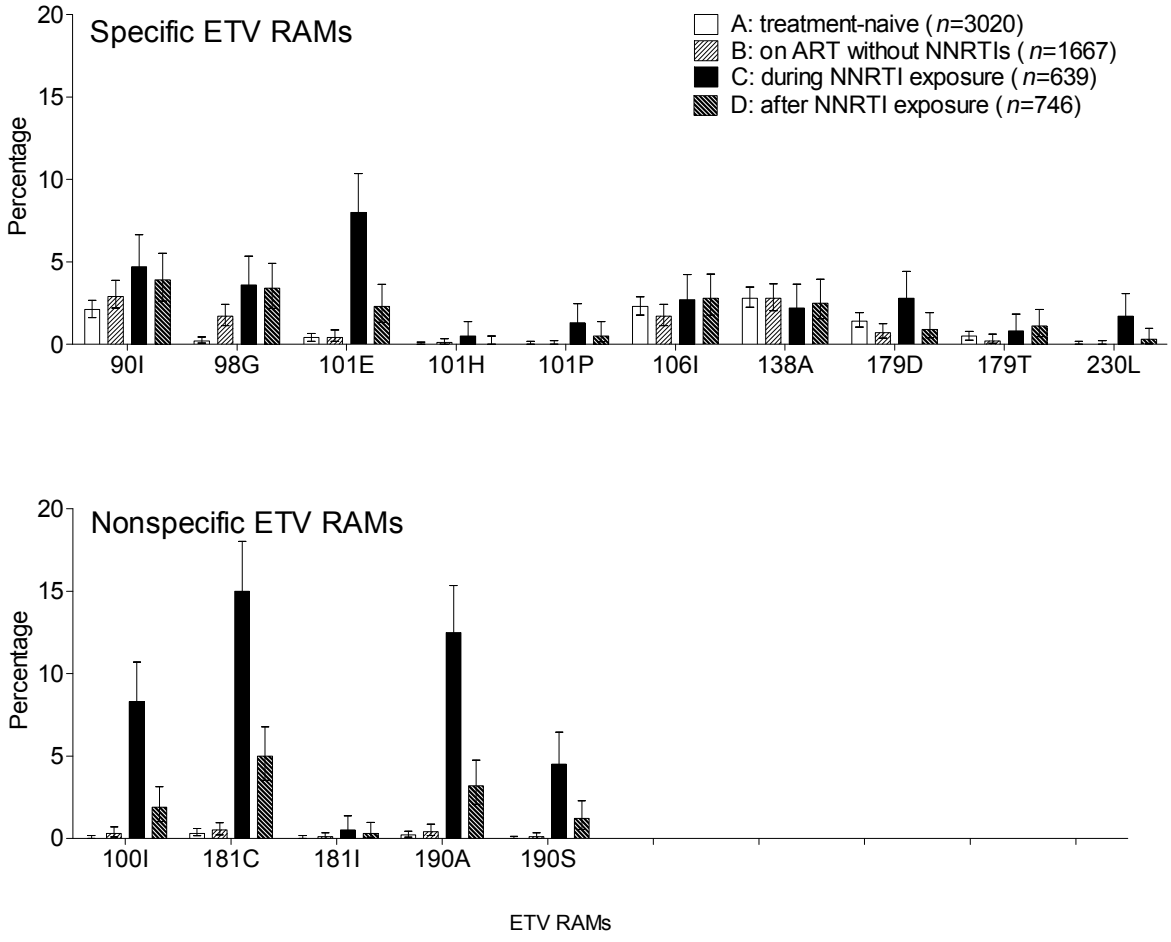

Figure 2. Percentage of etravirine resistance-associated mutations (ETV RAMs) based on genotypic resistance tests from treatment-naïve patients (group A), nonnucleoside reverse transcriptase inhibitor (NNRTI)-naïve patients (group B), patients exposed to NNRTIs [group C] and patients who had stopped treatment with NNRTIs (group D). The following ETV RAMs did not occur: 179F and 181V. ART, antiretroviral therapy

ETV RAMs in patients experiencing virological failure with EFV or NVP

We further focused our analysis on patients who had failed NVP- or EFV-containing therapies. In total, 258 GRTs were performed in patients with a virological failure, 182 (70.5%) of whom were exposed to EFV and 76 (29.5%) to NVP.

Frequencies of ETV-specific RAMs did not differ markedly according to exposure to EFV or NVP: V90I (4.9% on EFV *vs.* 5.3% on NVP, *P*=1.000), A98G (5.5% *vs.* 6.6%, *P*=0.773), K101E (8.2% *vs.* 11.8%, *P*=0.357), K101H (0.5% *vs.* 1.3%, *P*=0.503), K101P (3.3% *vs.* 0%, *P*=0.184), V106I (2.7% *vs.* 3.9%, *P*=0.697), E138A (1.6% *vs.* 5.5%, *P*=0.200), V179D (4.4% *vs.* 1.3%, *P*=0.289), V179T (0.5% *vs.* 1.3%, *P*=0.503) and M230L (3.8% *vs.* 0%, *P*=0.109). Certain nonspecific ETV mutations, however, preferentially occurred on treatment with specific NNRTIs, such as L100I (14.8% on EFV *vs.* 1.3% on NVP, *P*=0.001), Y181C (9.3% on EFV vs. 28.9% on NVP, *P*<0.001), and G190A (10.4% *vs.* 22.4%, *P*=0.017). The mutations Y181I (0% on EFV *vs.* 1.3% on NVP, *P*=0.295) and G190S (8.8% on EFV and 3.9% on NVP, *P*=0.203) occurred at similar frequencies in the two treatment groups.

Persistence of ETV RAMs following exposure to EFV or NVP

To analyse the persistence of ETV RAMs, we selected patients with a virological failure on EFV or NVP who had received a GRT during exposure to NNRTI and a later GRT performed on a therapy not containing NNRTI or off treatment (n=87). The median time between stop of NNRTIs and the second GRT was 897 days [interquartile range (IQR): 356-1555 days].

The prevalence of one or more ETV RAMs significantly decreased after cessation of exposure to NNRTIs from 51.7% (CI 40.8-62.6%) to 34.5% (CI 24.6-45.4%, *P*=0.032). The appearance of ETV RAMs tended to be less frequent in patients who had stopped ART (25%, CI 7.3-52.4%, *n*=16) compared with patients who had continued ART without NNRTIs (36.6%, CI 25.5-48.9%, *n*=71). Overall, 55.6% (CI 40.0-70.4%) of patients had lost at least one ETV RAM after the stop of NNRTIs. As shown in Figure 3, the frequency of almost all ETV RAM decreased after stop of NNRTI drugs.

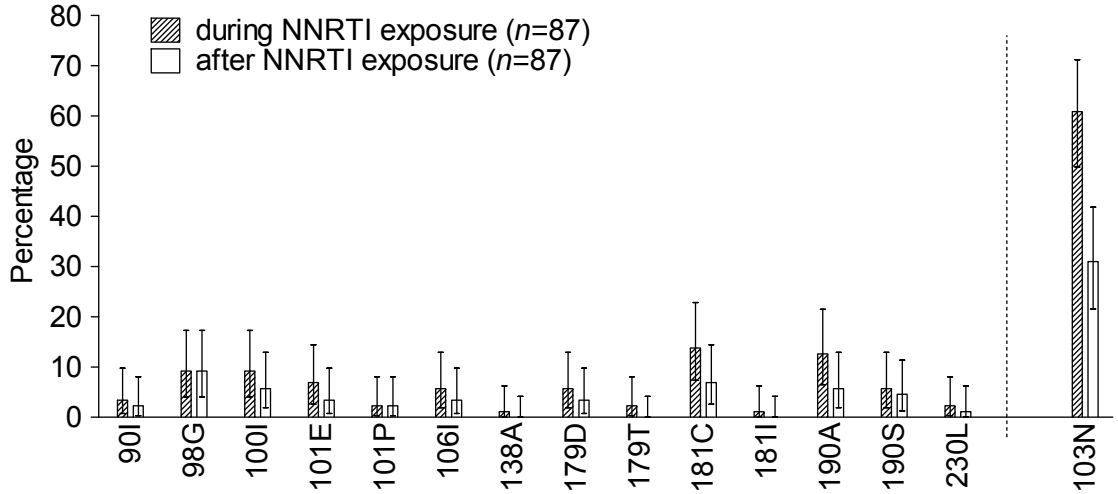

Figure 3. Changes in frequency of etravirine resistance-associated mutations (ETV RAMs) and K103N after discontinuation of nonnucleoside reverse transcriptase inhibitor (NNRTI) therapy. Patients with two genotypic resistance tests (GRTs), one performed during exposure to NNRTIs and a second performed at least 30 days after discontinuation of NNRTI treatment, were included (*n*=87). For comparison, the NNRTI mutation K103N is listed. K103N does not affect the antiviral activity of ETV, but is known to disappear after discontinuation of NNRTI treatment.[28]

Eligibility for ETV in the SHCS population based on different algorithms

We further aimed to determine the potential eligibility for treatment with ETV for 6072 GRTs performed based on the Stanford algorithm. Overall, ETV was considered fully active in 4599 (98.1%, CI 97.7-98.5%) NNRTI-naïve patients and in 518 (69.4%, CI 66.1-72.8%) patients with past NNRTI experience, but only in 262 (41.0%, CI 37.2-44.8%) patients with GRTs while being exposed to NNRTI.

For further analysis, we focused on patients with GRTs obtained while receiving EFV/NVP. Of all four interpretation methods the Stanford algorithm predicted the highest proportion of intermediate/full resistance in GRTs (56.2%, CI 52.2-60.1%). The predictions obtained with the IAS-USA recommendations (44.7%, CI 40.8-48.7%), Rega 7.1 algorithm (23.3%, CI 20.1-26.8%) and the ANRS algorithm (3.8%, CI 2.5-5.6%) were generally much lower (Fig. 4). The predicted frequency of full resistance was comparable across interpretation methods (Stanford: 3.8%, CI 2.5-5.6%; IAS-USA: 4.4%, CI 3.0-6.3%; Rega: 5.7%, CI 4.0-7.8%), with the exception of the ANRS algorithm, which classified 0.5% (CI 0.1-1.4%) of the samples as fully resistant. As shown in Figure 4, taking either EFV or NVP did not have differential effect on the estimated antiviral activity of ETV.

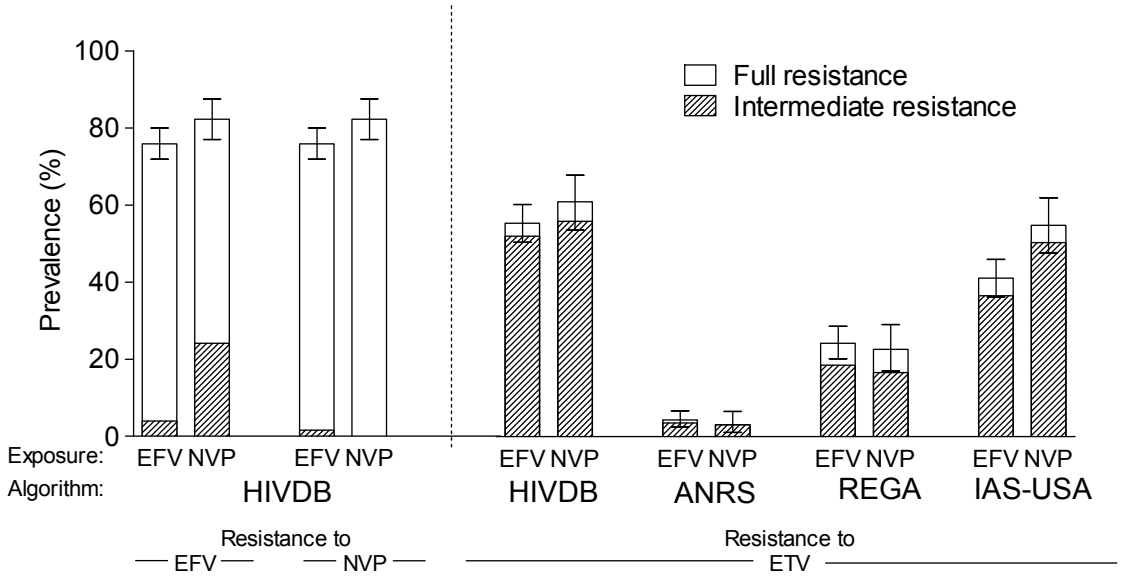

Figure 4. Proportion of genotypic resistance tests (GRTs) indicating intermediate or full resistance against etravirine (ETV), efavirenz (EFV) or nevirapine (NVP) according to different interpretation algorithms. The analysis was stratified by whether the patient was receiving EFV (*n*=428) or NVP (*n*=203) at the time of sampling. ANRS, Agence Nationale de Recherches sur le Sida et les hépatites virales; HIVDB, Stanford HIV Drug Resistance Data Base; IAS-USA, International AIDS Society-USA

Transmission of ETV drug resistance mutations

We analysed the prevalence of ETV RAMs in 763 GRTs in recently infected, treatment-naïve patients.[22] The prevalences of specific and nonspecific ETV mutations were 9.0% (CI 7.1-11.3%) and 0.9% (CI 0.3-1.9%), respectively, without any significant time trends (by the Cochran-Armitage test, not shown). Of note, the ETV RAMs V90I, A98G, E138A and V179D/T were detected in five of 51 (9.8%) plasma samples obtained before 1998, when NNRTIs were not yet registered in Switzerland. Thus, the detection of ETV RAMs in recently infected patients most probably reflects polymorphism rather than transmitted RAMs.

Estimated benefit of ETV in patients with different effective background regimen

Stratified by the GSS of the optimized background regimen, we aimed to identify the percentage of patients exposed to NNRTIs (group C) with a virus fully susceptible to ETV. The GSS of an optimized background treatment regimen was defined based on cumulative information from GRTs and consisting of the highest scoring of two NRTIs and one boosted PI. Based on the Stanford algorithm, only 22.9% (CI 15.4-32.0%) of samples with little residual antiviral activity of the background treatment (GSS<2) were fully susceptible to ETV, whereas significantly more viruses were susceptible (45.9%, CI 41.6-50.3%, *P*<0.001) in samples with more potent backbone regimens

(GSS≥2), indicating that the additional benefit of ETV may be limited for those in most need. As shown in Figure 5, other algorithms predicted higher benefits of ETV, however, and the frequency of patients with fully susceptible virus did not correlate with the magnitude of the GSS for the background regimens (ANRS algorithm, range 86.7-98.3%; REGA algorithm, range 60.0-83.8%). Of note, 81.9-84.9% of patients achieved a GSS of ≥2 for the background regimen.

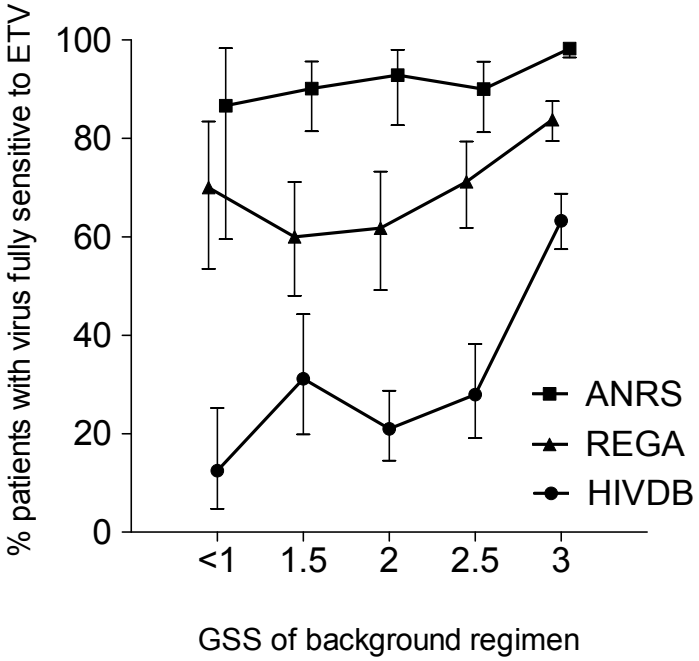

Figure 5: Proportion of patients with a genotypic resistance test performed while receiving nevirapine (NVP) or efavirenz (EFV) (*n*=631) and with a virus fully susceptible to etravirine (ETV). The analysis was stratified by a cumulative genotypic sensitivity score (GSS) for a hypothetical optimized background regimen consisting of two nucleoside reverse transcriptase inhibitor (NRTIs) and one protease inhibitor (PI). A GSS of 0 indicates full resistance and 1 indicates full susceptibility. ANRS, Agence Nationale de Recherches sur le Sida et les hépatites virales; HIVDB, Stanford HIV Drug Resistance Data Base; IAS-USA, International AIDS Society-USA

Clinical response

An intent-to-treat analysis (*n*=71) for the week 24 response showed an overall viral suppression rate of 71.8% (CI 59.9-81.9%). The presence of L100I, K101E/H/P or Y181C/I/V was more predictive for the short-term outcome (viral load <50 copies/mL after 24 weeks follow-up) than the GSS calculated using the Stanford algorithm. Patients with one of the above-mentioned mutations had a suppression rate of 52.9% (CI 27.8-77.1%) compared with 77.8% (64.4-88.0%) when these mutations were absent (*P*=0.065). In contrast, the GSS for ETV as estimated using the Stanford algorithm was not predictive for a virological response: response rates for fully susceptible viruses were 71.4% (CI 51.3-86.8%) compared with 72.1% (CI 56.3-

84.7%, *P*=1.000) for those with intermediate/full resistance. This tendency was confirmed, by a multivariable logistic regression including HIV RNA at the start of ETV treatment, the GSS of the background regimen and the presence of L100I, K101E/H/P, or Y181C/I/V. Failure to achieve undetectable HIV RNA was better predicted by the presence of one of the abovementioned mutations [odds ratio (OR): 2.8, CI 0.8-9.4] than by the Stanford algorithm (OR for intermediate/fully ETV-resistant HIV 0.7, CI 0.2-2.2).

Discussion

In this data set from a highly representative cohort study, the prevalence of ETV RAMs was higher in NNRTI-treated patients compared with NNRTI-naïve patients. ETV RAMs were lost at a relatively high rate once NNRTI drugs were stopped. Uncertainty remains for predictions of ETV activity, because analyses with different algorithms led to widely varying results.

The prevalence of ETV RAMs was 44.9% in patients tested while receiving NNRTIs, and 51.9% in patients failing on NNRTIs. Other studies have reported higher prevalence estimates of ETV RAMs in patients failing on NNRTIs, ranging between 61.7% and 74.1%.[29, 30] Also, the occurrence of three or more ETV RAMs was less common in our study.[12, 30, 31] The most likely explanation for the lower frequency of ETV mutations found in the SHCS might be that treatment failures in the SHCS were managed aggressively early on and that NNRTIs were rarely maintained in failing drug regimens,[15, 21] even when no other options were available. Two ETV mutations were associated with the largest impact on response, namely Y181I and Y181V.[32] The former mutation was rare and the later did not appear in our study sample. In addition, four mutations were also associated with a higher negative impact on response and their prevalence was increased with exposure to NNRTI treatment, namely L100I (8.3%), K101P (1.3%), Y181C (15.0%) and M230L (1.7%). It should be mentioned, that the prevalence of K101E increased under NNRTI treatment, even though K101E is not associated with NNRTI resistance.[32]

The fact that certain ETV-specific IAS-USA mutations such as 90I or 106I were already present at relative high frequencies in samples from treatment-naïve patients and their persistence in longitudinal samples suggests that these represent polymorphisms. This hypothesis is further strongly supported by the high frequency of transmitted ETV RAMs in our large study group with documented recent infection,

especially as the prevalence of ETV RAMs did not differ before and after registration of NNRTIs in Switzerland. Whether these polymorphisms really have an impact on ETV activity in clinical practice has to be further investigated in datasets with longer follow-up.

It should be noted that ETV RAMs that also affect other approved NNRTIs were lost at a relatively high rate once NNRTI drugs were stopped, as our analysis of GRTs performed on and off treatment containing NNRTIs has demonstrated. Overall, 55.6% of patients with sequential GRTs lost at least one ETV RAM, and hence GRTs performed distantly from NNRTI-containing therapy should be interpreted with caution because of the possible presence of minor viral variants with drug resistance mutations.

Overall, 631 GRTs were performed while patients were receiving EFV (*n*=428) or NVP (*n*=203). When analysed with Stanford algorithm, 67.5% of GRTs indicated full resistance to EFV and 76.9% to NVP, whereas full resistance against ETV was observed in 3.8% of GRTs. Full susceptibility to ETV was only predicted for 43.8% on EFV/NVP by the Stanford algorithm, suggesting considerable cross-resistance of ETV with approved NNRTIs. However, other interpretation methods such as ANRS and Rega or IAS-USA, gave less weight to the potential overlap of resistance mutations between ETV and EFV/NVP, thus yielding higher predictions for the proportion of viruses fully susceptible to ETV. Therefore, depending on interpretation methods used, we estimated that 43.8-96.2% of patients with a GRT performed while receiving potent NNRTI drug would be unaffected by the presence of resistance mutations and 3.3-54.9% could still get a partial benefit from treatment with ETV.

This low concordance among interpretation methods for predicting antiviral activity of ETV reflects the current uncertainty about how much the resistance profiles of ETV and EFV/NVP are overlapping. Moreover, the set of mutations that may affect viral response to ETV have not yet been clearly defined outside of the Duett studies.[9, 10] All these issues are highly relevant for decision-making in clinical practice, and further studies are warranted.

ETV is currently approved for use in salvage treatments. The application of a novel antiretroviral drug is especially interesting in patients with previous virological failure on NNRTIs and few alternative treatment possibilities. Depending on interpretation algorithm, our calculations showed that the effectiveness of ETV decreased almost

linearly in highly treatment-experienced patients with decreasing potency of an optimized background regimen.

The presence of major ETV IAS-USA mutations (L100I, K101E/H/P, Y181C/I/V) reduced the treatment response at week 24. The occurrence of these mutations was more predictive for clinical outcome than the Stanford algorithm. Although our sample size was relatively small for this analysis (*n*=71) a recent study investigating short-term responses at week 8 in a larger number of patients (n=243) supports our findings.[33]

Because of its good tolerability, a further potential application for ETV could be in patients with intolerance to EFV and NVP. In our study sample, 11% of patients had to cease treatment within 90 days of NNRTI initiation because of intolerance. ETV may be a safer alternative for these patients, because adverse events such as rashes are less severe and their incidence is lower compared with EFV treatment[8].

This study has some limitations. Not all GRTs in ART experienced patients were performed because of virological failures. We repeated the main analyses for those patients who failed NNRTI-treatment virologically, with virological failure defined as two consecutive on-treatment viral load measurements >500 copies/mL after at least 180 days of treatment. Of note, our results did not change. Moreover, as sequential GRTs were available for relatively few ART-exposed patients, eligibility may have been somewhat overestimated. However, the SHCS drug resistance database generally has a very high degree of completeness, with GRTs for more than 65% of all patients with a history of virological failures [von Wyl and Günthard, unpublished data].

In conclusion, here we clearly demonstrate that ETV RAMs in drug-naïve patients mainly reflect polymorphisms and probable do not confer high levels of resistance to ETV by themselves. Moreover, we have shown that only small differences exist in the prevalence of ETV RAMs among subtypes. Most importantly, caution is needed for the interpretation of GRTs performed off NNRTI treatment, because the frequency of ETV RAMs diminishes if the selection pressure is removed. Furthermore, considerable uncertainty remains in predicting the antiviral activity of ETV, underlined by widely discrepant results obtained using different interpretation systems. The newly defined weighted IAS-USA ETV mutations, however, were associated with enhanced clinical relevance when compared to the nonweighted mutations. Thus, the presence of these weighted mutations should particularly caution clinicians to use

ETV, in particular, in patients with extensive multidrug resistance. In this patient group, the predicted activity of ETV was the lowest, thus limiting the use of ETV in those patients most in need.

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Chapter 2

Implementation of raltegravir in routine clinical practice

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Implementation of raltegravir in routine clinical practice: selection criteria for choosing this drug, virologic response rates and characteristics of failures

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AUS contributed to the study design, performed the statistical analysis and drafted the article.

Abstract

Background

Raltegravir (RAL) achieved remarkable virological suppression rates in randomized-clinical trials, but today efficacy data and factors for treatment failures in a routine clinical care setting are limited.

Methods

First, factors associated with a switch to RAL were identified with a logistic regression including patients from the Swiss HIV Cohort Study (SHCS) with a history of 3 class failure (*n*=423). Second, predictors for virological outcome were identified in an intent-to-treat analysis including all patients who received RAL. Last observation carried forward imputation was used to determine week 24 response rate (HIV-1 RNA<50 copies/mL).

Results

The predominant factor associated with a switch to RAL in patients with suppressed baseline RNA was a regimen containing enfuvirtide [odds ratio: 41.9 (95%CI: 11.6-151.6)]. Efficacy analysis showed an overall response rate of 80.9% (152/188), whereas 71.8% (84/117) and 95.8% (68/71) showed viral suppression when stratified for detectable and undetectable RNA at baseline, respectively. Overall CD4 cell counts increased significantly by 42 cells/µl (*P*<0.001). Characteristics of failures were a genotypic sensitivity score of the background regimen ≤1, very low RAL plasma concentrations, poor adherence and high viral load at baseline.

Conclusions

Virological suppression rates in our routine clinical care setting were promising and comparable to data from previously published randomized-controlled trials.

Introduction

Currently, the strategy for HIV treatment is based on combination therapy to achieve durable viral suppression. Long-term use of HIV treatment can be jeopardized by the development of resistance and drug toxicity. Multi-drug resistance and cross-resistance to agents within the same drug class lead to restricted treatment options.[1-3] The introduction of the integrase inhibitor raltegravir (RAL) has broadened the treatment possibilities for highly treatment experienced patients considerably.[4-7]

In Switzerland, RAL has been registered since February 2008 for use only in highly antiretroviral treatment (ART) experienced patients with detectable HIV-1 RNA. Phase 2 and phase 3 clinical trials showed that RAL, together with an optimized background regimen, provided good HIV-1 suppression, in particular, in patients with triple-class drug failure and extensive drug resistance.[5, 6, 8] Success rates were comparable with what has been achieved in earlier salvage studies.[9-13] There are no major pharmacokinetic interactions known with other antiretroviral drugs, although atazanavir increases and tipranavir decreases the plasma concentration of RAL moderately.[14-18] RAL is the last option to achieve complete viral suppression for many highly treatment experienced patients. Due to its good tolerance and high efficacy, RAL may also be an option for treatment simplification, for example to circumvent a regimen including enfuvirtide (T20), which is difficult to administer and causes allergic injection site reactions,[19, 20] or potentially to reduce drug toxicity caused by other drugs, such as dyslipidemia or liver toxicity.

Using data from the Swiss HIV Cohort Study (SHCS), we aimed to identify the main factors associated with a change of the ART to a regimen containing RAL. We further analyzed the outcome of this switch in terms of sustained undetectable viral loads and CD4 cell count recovery after 24 weeks of treatment. For patients with incomplete viral suppression after 24 weeks of RAL treatment, we attempted to identify factors associated with suboptimal treatment response by assessing adherence, results from therapeutic drug monitoring and HIV drug resistance.

Methods

Patient selection and study design

The Swiss HIV cohort study (SHCS) is a nationwide, multicenter, clinic-based, highly representative cohort with continuous enrolment and at least semiannual study visits.[21] The study has been approved by ethical committees of all participating

institutions, and written informed consent has been obtained from all participants (ClinicalTrials.gov, #NCT00904644). For the present analysis, we included patients with at least one SHCS study visit after February 1, 2008, and patients who participated in the expanded access program before the registration of RAL in Switzerland. Two analyses with different study populations were performed; one analysis aimed to assess factors associated with the switch to RAL, and the second analysis was an efficacy study.

Patient selection for identification of factors associated with switching to RAL

We identified factors for switching the previous ART to a regimen containing RAL. Because in Switzerland RAL is only approved for use in highly treatment experienced patients, we included only individuals who had experienced triple class failure on nucleoside reverse transcriptase inhibitor (NRTI), nonnucleoside reverse transcriptase inhibitor (NNRTI), and protease inhibitor (PI). Virological failure was defined as viral rebound after previous suppression with 2 consecutive viral loads greater than 500 copies/mL or a single value greater than 500 copies/mL followed by a stop or a modification of the current therapy. In addition, we considered patients with evidence for triple class failure based on cumulative information from genotypic drug resistance testing, defined as the presence of at least 1 class-specific IAS-USA mutation against each of all 3 classes.[22]

Patient selection for efficacy analysis

Additionally, we aimed to study the efficacy of RAL, defined as an HIV-1 RNA<50 copies/mL after 24 weeks (window 20-28 weeks) of continuous treatment with RAL. Contrary to the first analysis, in the efficacy analysis we included all patients who had started a treatment with RAL, irrespective of their treatment history or resistance pattern to obtain most representative estimates for RAL efficacy in a routine clinical care setting.

Routinely performed measurements of RAL plasma levels were included in the study and compared to previous published levels, where logarithmic RAL plasma concentrations were approximately linear between the peak (1.7h) and 12h after administration. We considered plasma levels below the lower 90% confidence interval (CI) as too low.[23] Drug levels were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS).[24]

Statistical method

Baseline was defined as the start of the first ART including RAL. Multivariable logistic regression was used to identify predictors for switching to a treatment containing RAL. The following baseline characteristics were considered in the model: sex, ethnicity, age, risk group, CD4 cell count, number of drugs in the last treatment, the presence of T20 in the last regimen, self-reported adherence, genotypic sensitivity score (GSS) of the optimized background regimen, lipid profile [triglycerides, high-density lipoprotein (HDL), cholesterol, total cholesterol], and the Framingham risk score.[25] Predictors were included in the multivariable analysis if the univariable *P* value was <0.2 and sex, ethnicity, risk group and age, irrespective of its *P* value. As an a priori hypothesis we postulated that for patients with detectable HIV-1 RNA at start of RAL the main factor for RAL initiation may be to achieve viral suppression, whereas switches to RAL in patients with prior undetectable HIV-1 RNA may be driven by the wish for treatment simplification and toxicity concerns. We therefore developed 2 separate logistic regression models for patients with undetectable baseline HIV-1 RNA (switch patients) and patients with detectable baseline viral load (salvage patients).

An intent-to-treat analysis was performed to determine RAL efficacy. Two strategies were used to account for missing information on endpoints: missing values were considered failures (m = f), or we carried forward the last HIV-1 RNA measurement obtained before week 20 to impute the week 24 HIV-1 RNA [last observation carried forward (LOCF)]. Factors for a virological nonresponse to RAL after 24 weeks were assessed with logistic regression models using the same covariables for adjustment as in the prediction model for switching to RAL. In addition, we also included baseline HIV-1 RNA, CD4 nadir, coadministration of boosted PIs, GSS of the background regimen and central nervous system (CNS) penetration effectiveness (CPE) rank of the background regimen as covariables. The GSS was calculated based on the Stanford algorithm (version 6.0.1; HIV Drug Resistance Database, Stanford, CA) for all approved NRTI, NNRTI, and PI drugs. Viral susceptibility to T20 and maraviroc (MVC) was considered to be intact if these drugs had not been included in a previous failing regimen. Genotypic data were obtained from the SHCS resistance database which contains all genotypic HIV resistance tests performed by the 4 authorized laboratories in Switzerland, stored in SmartGene's (Zug, Switzerland) Integrated Database Network System (IDNS version 3.5.4).[26]

A CPE of 0, 0.5 or 1 indicated low, intermediate or high CNS penetration, respectively. The CPE of MVC was estimated as 1 and the CPE of etravirine (ETV) as 0.5.[27-29]

Mixed-effects linear regression was performed to estimate the increase in CD4 cell count after week 24.

Statistical analysis was performed with Stata 10 SE (StataCorp, College Station, TX, USA). The level of significance was set at $P<0.05$.

Results

Factors associated with a switch to RAL

We identified 423 patients who had experienced triple class failures and were actively participating in the SHCS in February 2008 of whom 238/423 (56.3%) had undetectable viral loads at the last HIV-1 RNA measurement and 185/423 (43.7%) had detectable viral loads, respectively. In total, 123 (29.1%) patients changed the ART to a regimen containing RAL, 48/238 (20.2%) of whom had undetectable and 75/185 (40.5%) had detectable viral loads (HIV-1 RNA>50 copies/mL) at the time of treatment change. Mentionable, 48/123 (39.0%) of patients who started RAL had an undetectable viral load.

As shown in Table 1, in patients with undetectable viral load at baseline (n=238) the multivariable model indicated that low CD4 cell counts (<200cells/µL), exposure to T20 and increased triglycerides (>2.3 mmol/L) were important factors to change the current ART to a RAL-containing regimen. Heterosexual patients received RAL less likely compared with homosexual men. Additionally, age, more than 5 drugs in the last regimen, decreased HDL cholesterol (<0.9 mmol/L), increased Framingham score and low GSS of the background regimen were significantly associated with RAL administration in the univariable model.

Table 1. Univariable and multivariable logistic regression to identify factors associated with a switch to RAL (n=48) in patients with triple-class failure (n=238) and undetectable HIV-1 RNA (<50 copies/mL).

		Univariable analysis			Multivariable analysis		
Characteristics	**N (%) (on RAL/TCF)**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Sex							
Male	40/172 (23.3)	Ref					
Female	8/66 (12.1)	0.5	(0.2-1.0)	0.060	2.3	(0.6-9.5)	0.251
Ethnicity							
White	46/206 (22.3)	Ref					
Other	2/32 (6.3)	0.2	(0.1-1.0)	0.051	1.0	(0.1-7.3)	0.990
Risk							
Homosexual	28/98 (28.6)	Ref					
Intravenous drug use	9/42 (21.4)	0.7	(0.3-1.6)	0.381	1.2	(0.3-4.9)	0.800
Heterosexual	9/86 (10.5)	0.3	(0.1-0.7)	**0.003**	0.2	(0.1-0.8)	**0.026**
Other	2/12 (16.7)	0.5	(0.1-2.4)	0.390	1.5	(0.2-12.1)	0.700
Age per year older		1.5	(1.1-2.0)	**0.009**	1.4	(0.8-2.2)	0.227
CD4 cell count							
≥200 cells/μL	40/222 (18.0)	Ref					
<200 cells/μL	8/16 (50.0)	4.6	(1.6-12.8)	**0.004**	5.4	(1.0-29.0)	**0.050**
No. drugs in last regimen							
0-3	8/74 (10.8)	Ref					
4-5	28/133 (21.1)	2.2	(0.9-5.1)	0.067	0.5	(0.1-1.7)	0.254
Over 5	12/31 (38.7)	5.2	(1.9-14.6)	**0.002**	0.4	(0.1-1.8)	0.214
Self-reported adherence							
Never missed a dose	39/190 (20.5)	Ref					
1 dose per month	7/37 (18.9)	0.9	(0.4-2.2)	0.824			
1 dose per week	2/11 (18.2)	0.9	(0.2-4.1)	0.851			
Exposure to T20 in last regimen							
Unexposed	10/175 (5.7)	Ref					
Exposed	38/63 (60.3)	25.1	(11.0-56.6)	**<0.001**	41.9	(11.6-151.6)	**<0.001**
Total cholesterol							
Normal	39/191 (20.4)	Ref					
High (>6.2mmol/L)	9/47 (19.1)	0.9	(0.4-2.1)	0.846			
HDL cholesterol							
Normal	28/168 (16.7)	Ref					
Low (<0.9mmol/L)	20/70 (28.6)	2.0	(1.0-3.9)	**0.039**	0.7	(0.2-2.0)	0.473
Triglycerides							
Normal	16/134 (11.9)	Ref					
High (> 2.3 mmol/L)	32/104 (30.8)	3.3	(1.7-6.4)	**<0.001**	4.0	(1.5-11.0)	**0.006**
Framingham score per 10% increase		1.9	(1.2-2.8)	**0.004**	0.8	(0.4-1.5)	0.439
GSS optimized background regimen							
GSS <2	42/149 (27.7)	Ref					
GSS ≥2	6/80 (8.6)	0.2	(0.1-0.5)	**0.001**	0.4	(0.1 -1.1)	0.084
Unknown	0/9 (0)	-	-	-	-	-	-

Bold values indicate *P* <0.05
TCF patients who experienced triple class failure, OR odds ratio.

In patients with detectable viral load, the multivariable model showed that exposure to T20 in the previous regimen, low CD4 cell counts, self-reported adherence and low GSS of the optimized background regimen were associated with the decision to include RAL in the salvage ART (Table 2). Intravenous drug users received RAL less likely compared to homosexual men. Additionally, in the univariable model also heterosexual patients received RAL less likely. In contrast to patients with suppressed viremia, lipid abnormalities were not correlated with the change in patients with detectable viral load.

Efficacy analysis

We performed an intent-to-treat analysis to investigate whether the change to RAL was successful in patients with detectable (salvage patients) or suppressed (switch patients) viral load at baseline, respectively. In total, 243 patients had started a regimen containing RAL since February 2008. Three (1.2%) patients were excluded because of missing baseline data and 52 patients were excluded, because RAL was started within less than 20 weeks before the cut-off date for this analysis (28 February 2009). Thus, for the efficacy analysis, we included 188 patients, of whom 184 still were on RAL after 24-week follow-up. A total of 117 patients were salvage patients and 71 switch patients. During follow-up, 4 patients interrupted ART for a median time of 25 days (range: 1-136) and four patients interrupted the intake of RAL for a median time of 57 days (range: 10-61), but all except 1 continued with a regimen including RAL later on. Additionally, 25 patients changed their background regimen during the follow-up. Further, 1 patient (0.5%) died because of AIDS, 1 committed suicide (0.5%) and 1 patient died of unknown reasons (0.5%) within the first 20 weeks of follow-up and 36/188 (19.2%) patients had no available HIV-1 RNA measurement within week 20 and 28 after RAL start.

Table 2. Univariable and multivariable logistic regression to identify factors associated with a switch to RAL (n=75) in patients with triple-class failure (n=185) and detectable HIV-1 RNA (≥50 copies/mL).

		Univariable analysis			Multivariable analysis		
Characteristics	**N (%) (on RAL/TCF)**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Sex							
Male	58/135 (43.0)	Ref			Ref		
Female	17/50 (34.0)	0.7	(0.3-1.3)	0.271	1.3	(0.4-4.0)	0.649
Ethnicity							
White	65/154 (42.2)	Ref			Ref		
Other	10/31 (32.3)	0.7	(0.3-1.5)	0.306	0.4	(0.1-1.5)	0.168
Risk							
Homosexual	47/86 (54.7)	Ref			Ref		
Intravenous drug use	4/23 (17.4)	0.2	(0.1-0.6)	**0.003**	0.1	(0.0-0.4)	**0.001**
Heterosexual	20/64 (31.3)	0.4	(0.2-0.7)	**0.005**	0.5	(0.2-1.4)	0.194
Other	4/12 (33.3)	0.4	(0.1-1.5)	0.176	0.4	(0.1-2.5)	0.338
Age per year older		1.2	(0.9-1.5)	0.232	0.8	(0.5-1.3)	0.458
CD4 cell count							
≥200 cells/μL	42/138 (30.4)	Ref			Ref		
<200 cells/μL	33/47 (70.2)	5.4	(2.6-11.1)	**<0.001**	9.2	(3.4-24.4)	**<0.001**
No. drugs in last regimen							
0-3	31/94 (33.0)	Ref			Ref		
4-5	36/79 (45.6)	1.7	(0.9-3.2)	0.091	1.3	(0.6-2.9)	0.575
Over 5	8/12 (66.7)	4.1	(1.1-14.5)	**0.031**	1.3	(0.2-6.8)	0.783
Self-reported adherence							
Never missed a dose	55/122 (45.1)	Ref			Ref		
1 dose per month	8/26 (30.8)	0.5	(0.2-1.3)	0.184	0.4	(0.1-1.5)	0.182
1 dose per week	8/32 (25.0)	0.4	(0.2-1.0)	**0.044**	0.3	(0.1-0.9)	**0.036**
Unknown	4/5 (80.0)	4.9	(0.5-44.9)	0.162	3.2	(0.2-68.2)	0.449
Exposure to T20 in last regimen							
Unexposed	46/142 (32.4)	Ref			Ref		
Exposed	29/43 (67.4)	4.3	(2.1-9.0)	**<0.001**	4.3	(1.6-11.7)	**0.004**
Total cholesterol							
Normal	65/163 (39.9)	Ref					
High (>6.2 mmol/L)	10/22 (45.5)	1.3	(0.5-3.1)	0.618			
HDL cholesterol							
Normal	43/118 (36.4)	Ref			Ref		
Low (<0.9 mmol/L)	32/67 (47.8)	1.6	(0.9-2.9)	0.133	1.0	(0.4-2.5)	0.915
Triglycerides							
Normal	42/105 (40.0)	Ref					
High (>2.3 mmol/L)	33/80 (41.3)	1.1	(0.6-1.9)	0.864	Ref		
Framingham score per 10% increase		1.6	(1.0-2.6)	0.051	1.1	(0.5-2.5)	0.781
GSS optimized background regimen							
GSS <2	61/115 (53.0)	Ref			Ref		
GSS ≥2	14/68 (20.6)	0.2	(0.1-0.5)	**<0.001**	0.3	(0.1-0.8)	**0.010**
Unknown	0/2 (0)	-	-	-	-	-	-

Bold values indicate *P* <0.05
TCF patients who experienced triple class failure, OR odds ratio.

In switch patients, the median number of drugs in the background regimen was 3 (range: 1-7). 40.9% had an NNRTI, 84.5% at least 1 NRTI and 60.1% a boosted PI. Unboosted PIs were coadministered in 5 (7.4%) cases. At baseline, 46.5% had T20, which was replaced in almost all patients (n=30, 90.9%). 22.5% were treated with ETV, the latest approved NNRTI, and 5.6% with MVC.

Salvage patients had a median number of 3 (range: 1-7) coadministered drugs. The background regimen included an NNRTI in 45.3% of the patients, an NRTI in 76.1%, a boosted PI in 70.9%. Unboosted PIs were rarely coadministered (6.8%). At baseline 16 (13.6%) patients were treated with T20, which was replaced in most cases (75%). The newer drugs, ETV and MVC were administered in 37.6% and 13.7%, respectively.

Many salvage and switch patients had a documented 3 class failure before starting RAL, 57.3% and 59.2%, respectively.

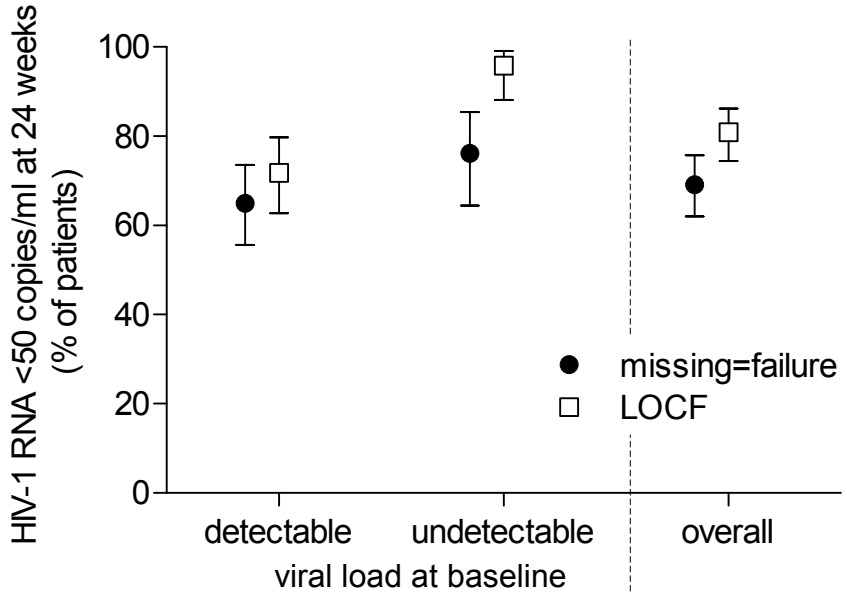

Figure 1. Percentage of patients with plasma HIV-1 RNA levels less than 50 copies/mL after 24 weeks of RAL combined with a background regimen. Percentages are shown for patients with undetectable (HIV-1 RNA <50 copies/mL), detectable HIV-1 RNA at baseline, and the overall response rate. Intent-to-treat analysis was performed for 2 imputation methods, missing equal failure [closed circle] and LOCF (n=188) [open square]. Error bars represent 95% CIs.

Salvage patients had median plasma HIV-1 RNA of 3.8 $\log_{10}$ copies/mL [interquartile range (IQR): 2.9-4.7] at baseline. Results from the intent-to-treat analysis are shown in Figure 1 according to the 2 imputation methods, which were missing equal failure (m = f) and LOCF. The overall week 24 response rates were 69.2% (m = f) and 80.9% (LOCF), respectively. Response rates for switch patients were 76.1% (m = f)

and 95.8% (LOCF), respectively. For salvage patients, the estimates were 65.0% (m = f) and 71.8% (LOCF), respectively. Median baseline CD4 cell counts at baseline were 226 cells/µL (IQR: 134-328) and 351 cells/µL (IQR: 252-483) for salvage and switch patients, respectively. The CD4 cell count increased within 24 weeks by 51 cells/µL (95% CI: 39-64, *P*<0.001) and by 22 cells/µL (95% CI: 7-37, *P*=0.003), respectively. The overall increase was 42 cells/ µL (95% CI: 32-52, *P*=<0.001).

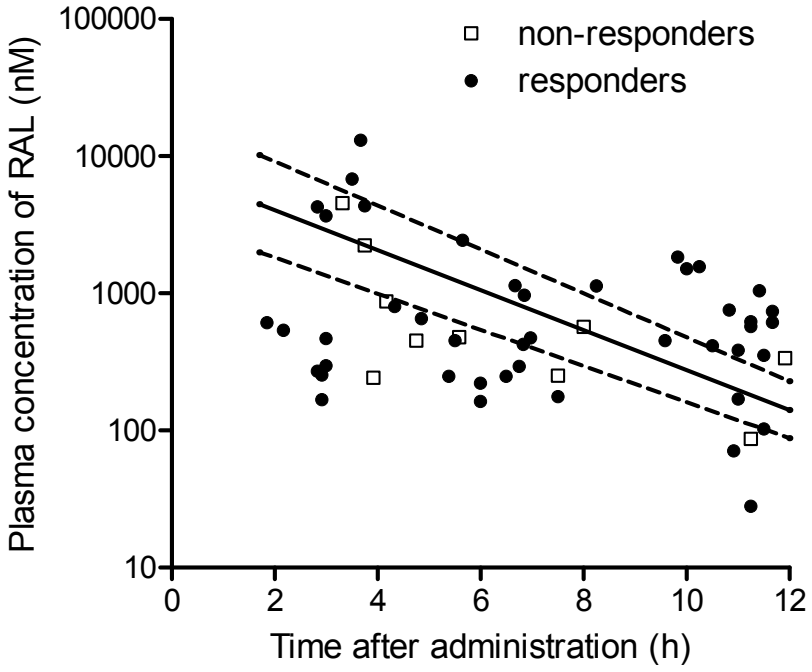

Figure 2. Plasma concentration of RAL after at least 10 days with twice daily doses of 400 mg. Patients with detectable [closed circle] (HIV-1 RNA ≥50 copies/mL) and undetectable HIV-1 RNA [open square] after 24 weeks follow-up were distinguished. Expected drug levels, mean [**—**] and upper and lower 90% confidence interval [--] were indicated, based on previous pharmacokinetic studies.[23]

Characteristics of failures

Univariable and multivariable logistic regressions (not shown) including risk group, ethnicity, sex, self-reported adherence, CPE, baseline HIV-1 RNA and the GSS of the background regimen did not show any significant factor for treatment success. The number of patients with failure to achieve undetectable viral loads at week 24 was low, (n=36, 33 salvage patients, 3 switch patients). As shown in table 3, characteristics for failure were identified descriptively. Patients classified as failures because of insufficient follow-up time (n=15) were excluded. Six of the 21 patients listed in the table (#5, #6, #14, #17, #18, #20) had a low GSS ≤1 of the background regimen, 6 had very low plasma concentrations of RAL (#1, #2, #7, #11, #12, #20),

Table 3. Patients (n=21) with a documented failure (HIV-1 RNA>50 copies/mL) at week 24 after RAL start

Patient	Treatment*		CD4 count (cells/µl)		Log HIV-1 RNA (copies/ml)		Self-reported adherence	RAL plasma concentration[+]	RAL mutations	GSS OBT[§]	CPE
	pre-RAL	on RAL	Baseline	Week 24	Baseline	Week 24					
#1	TDF RTV ETV FTC DRV	TDF RTV RAL FTC DRV	206	250	2.0	1.8	good	low	-	2	1.5
#2	SQV RTV DDI ZDV	T20 RAL DRV	591	462	3.4	1.7	good	low		2	0.5
#3	TDF T20 RTV DRV ZDV ABC 3TC	TDF T20 RTV RAL DRV ZDV ABC 3TC	121	156	2.9	3.5	poor	-		1.5	3
#4	LPV EFV ZDV 3TC	RAL LPV ZDV 3TC	683	477	2.0	2.1	good	normal		3	2.5
#5	TPV RTV ETV	RAL LPV ETV	413	575	3.1	2.0	good	-		0.5	1.5
#6	MVC/placebo[‡] TDF T20 ABC 3TC	RAL ETV 3TC	28	11	5.2	5.0	-	-	none	0.5	1
#7	TDF T20 FTC EFV	TDF RAL FTC EFV	606	769	0.0	1.7	good	low		2.5	1.5
#8	LPV FPV ABC	RTV RAL MVC ETV DRV	190	295	5.4	1.9	good	-		2.5	2
#9	RTV ATV ABC 3TC	RTV RAL ETV DRV	281	191	3.3	2.9	poor	normal	Q148R	2	1
#10	TDF NVP ABC 3TC	TDF RTV RAL ETV DRV ABC 3TC	64	194	5.2	3.0	good	normal		3	2.5
#11	T20 RTV ETV DRV ABC 3TC	RTV RAL ETV DRV ABC 3TC	116	30	4.1	5.4	poor	low	none	3	2.5
#12	TDF RTV FTC ATV	TDF RTV RAL ETV FTC DRV	396	711	4.8	2.0	good	low		2	2
#13	TPV TDF T20 RTV LPV ZDV ABC 3TC	TDF T20 RTV RAL FTC DRV	269	258	2.4	2.3	good	-		1.5	1.5
#14	TDF RTV FTC ATV	RTV RAL ATV	167	190	4.7	5.1	-	-		1	0.5
#15	TPV TDF DDI 3TC	TPV RTV RAL ETV DRV	14	63	5.2	4.7	poor	-	Y143C	1.5	1
#16		TDF RTV RAL FTC ATV	125	253	5.2	1.9	good	-		2	1.5
#17		TDF RAL EFV	284	320	4.6	4.3	good	-		0.5	0.5
#18	TDF NVP FTC	RAL ATV	28	170	4.2	3.2	poor	-		1	0.5
#19	NVP ZDV ABC 3TC	TDF RAL NVP	147	191	1.9	2.1	good	normal		2	1
#20	TPV TDF T20 RTV MVC ZDV ABC 3TC	TDF RTV RAL MVC ETV DRV ZDV 3TC	600	592	2.1	2.1	-	low		1	3.5
#21	TDF RTV FTC ATV	RTV RAL ETV DRV	592	650	2.2	1.8	good	-		1.5	1

*TDF, tenofovir; RT,V ritonavir; SQV, saquinavir; FTC, emtricitabine; DRV, darunavir; DDI, didanosine; ZDV, zidovudine; ABC, abacavir; 3TC, lamivudine; LPV, lopinavir; EFV, efavirenz; TPV, tipranavir; FPV, fosamprenavir; NVP, nevirapine; ATV, atazanavir. [+]based on pharmacokinetic studies [23] ; [§]GSS OBT genotypic sensitivity score of the optimized background treatment, [‡]blinded clinical trial, code not broken yet.

five patients had a poor self-reported adherence (#3, #9, #11, #15, #18), and 5 patients had a very high HIV-1 RNA (>100,000 copies/mL) at baseline (#6, #8, #10, #15, #16) and for 4 patients there was no clear explanation for the failure (#4, #13, #19, #21). Four patients had a genotypic resistance test performed while failing on RAL 1 patient had the Q148R mutation and one the Y143C mutation; the other two patients had no known RAL resistance-associated mutation (Table 3).

Drug levels were measured for 54 patients, and 46.3% had a lower RAL plasma concentration than expected. Six of 25 (24.0%) patients with low and 4/29 (13.8%) with normal RAL plasma concentration were not suppressed after 24 weeks follow-up. However, drug levels showed very large inter-patient variation (Fig. 2).

Discussion

In our study population, the main reasons to include RAL in an antiretroviral regimen were low CD4 cell counts and replacement of T20. Treatment success, defined as an HIV-1 RNA<50 copies/mL after 24 weeks of follow-up, was analyzed with an intent-to-treat approach using LOCF imputation and yielded estimates of 95.8% for switch patients and 71.8% for salvage patients.

Our study reflects the introduction of the newly available drug RAL into a routine clinical care setting within the SHCS, a highly representative study for the Swiss HIV-infected population. 74% of all NRTI compounds sold within Switzerland are prescribed within the SHCS (B. Ledergerber, personal communication). Despite the knowledge that RAL has almost no interaction potential with other drugs and has a very favourable lipid profile, RAL received a very restricted approval by the Swiss health authorities, most likely because RAL prices are high compared with most licensed drugs. Interestingly, however, our study clearly demonstrates that 39% of RAL use was not within the approved indication (replicating virus), and among those the main reason for switching to RAL was the replacement of T20 (60.3%). Thus, health insurers in Switzerland were quite supportive with regards to approving the formal requests that had to be individually written for each patient by the treating physician if a drug is used outside the approved indication. As an a priori hypothesis, we postulated that for patients with detectable HIV-1 RNA at start of RAL the main factor for RAL initiation may be a low GSS of the background regimen, whereas switches to RAL in patients with prior undetectable HIV-1 RNA may be driven by the wish for treatment simplification and toxicity concerns. This hypothesis was partially

confirmed by our results. Low GSS of the optimized background regimen and good self-reported adherence were associated with switching to RAL in patients with detectable viral load, whereas increased triglycerides were significantly associated in patients with undetectable viral load. In both models, exposure to T20, low CD4 cell counts were positively associated with the treatment change, being an intravenous drug user was negatively associated.

This is a comprehensive observational study presenting efficacy data of RAL in routine clinical practice for salvage and switch patients. We implemented an intent-to-treat analysis with 2 strategies to handle missing data, which were to consider patients with missing endpoints as failures and to carry the LOCF and yielded an overall efficacy of 69.2% and 80.9%, respectively. In this observational dataset, results from the LOCF imputation method are more meaningful, because the rate of missing values was quite high (19.5%). The efficacy when performing the LOCF imputation were slightly lower compared to the efficacy estimates including only patients with a HIV-1 RNA measurement between week 20 and 28 were included (86.1%), which were 98.2% and 79.2% (data not shown) for switch and salvage patients, respectively.

Based on the data from LOCF imputation, a switch from a previous treatment to a regimen containing RAL in patients with suppressed viral loads seemed highly effective because 95.8% of these study subjects had an HIV-1 RNA <50 copies/mL at week 24. Only 3 highly treatment experienced patients had detectable viral loads at week 24. This is comparable with results from a study by Harris et al, which showed that 34/35 patients who switched from RAL to T20 were virologically suppressed after 7-month follow-up time.[20, 30] The SWITCHMRK trials studied the replacement of lopinavir/ritanovir-based regimens to a RAL-based regimen and obtained slightly lower efficacy estimates compared to the switch patients in our study, however 60% of our patients were kept on a boosted PI containing regimen in contrast to the SWITCHMRK trials.[30] A replacement of T20 with RAL would have considerable advantages for patients concerning manageability and tolerance of the treatment.

After 24 weeks of follow-up, 71.8% of salvage patients had no detectable viral replication, which is comparable to previous published phase 3 randomised-control trials (BENCHMARK 1, 2) where approximately 78% of patients attained suppression of viral replication at week 16.[5, 6]

A careful in depth analysis of factors potentially associated with RAL failure did not reveal clear predictors for failure. In the first place, this may be surprising, however, due to the low overall number of documented failures (n=21) in our study and due to multifactorial reasons for treatment failures in ART this finding makes sense. Of note is our observation of low RAL drug levels in more than 46% of patients, which clearly demonstrates that more knowledge is needed for useful interpretation of RAL drug levels in the context of virological response. Although low RAL drug levels did not predict virological failure in our and in other studies it has to be noted that 60% of our failures indeed showed very low RAL drug levels compared to 43% of patients with successful viral suppression.[5, 6] To date it is not known how well RAL penetrates into the central nervous system. Thus, we speculated that if RAL would not penetrate well, failures would potentially be associated with a lower CPE score of the background regimen compared with nonfailures. However, our results do not support this hypothesis.

This study has some limitations. At this point we looked at 24-week efficacy, and in the future also long-term efficacy and safety of RAL in clinical practice will need to be further analyzed. This is an observational study, treatments were not randomized and patients had very different treatment histories, and as in all observational studies, residual confounding can not be excluded. The particular indication to measure drug levels was unknown and the selection of the 54 drug levels measured might be biased. A strength of this study is the very comprehensive assessment of clinical and laboratory data, including adherence, genotypic resistance data and data from therapeutic drug monitoring in a highly representative cohort.

In summary, this study demonstrated very good week 24 efficacy of RAL in patients with previous triple-class failure with detectable or undetectable viral load at baseline. The main reason for the selection of RAL in patients with undetectable viral load was to replace T20, although to date only little is known about the effectiveness of such changes. Moreover, RAL plasma concentration levels were lower than expected in a large proportion of patients but failed to predict clinical outcomes in our statistical analyses. Further studies are needed to analyze long-term efficacy of RAL.

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The members of the Swiss HIV Cohort Study are Battegay M, Bernasconi E, Böni J, Bucher HC, Bürgisser P, Calmy A, Cattacin S, Cavassini M, Dubs R, Egger M, Elzi L, Fischer M, Flepp M, Fontana A, Francioli P (President of the SHCS), Furrer H (Chairman of the Clinical and Laboratory Committee), Fux CA, Gorgievski M, Günthard HF (Chairman of the Scientific Board), Hirsch HH, Hirschel B, Hösli I, Kahlert C, Kaiser L, Karrer U, Kind C, Klimkait T, Ledergerber B, Martinetti G, Müller N, Nadal D, Paccaud F, Pantaleo G, Rauch A, Regenass S, Rickenbach M (Head of Data Center), Rudin C (Chairman of the Mother & Child Substudy), Schmid P,Schultze D, Schüpbach J, Speck R, de Tejada BM, Taffé P, Telenti A, Trkola A, Vernazza P, Weber R, Yerly S.

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Chapter 3

NRTIs in salvage treatment

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Viral suppression rates in salvage treatment with raltegravir improved with the administration of genotypic partially active or inactive nucleoside/tide reverse transcriptase inhibitors

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AUS contributed to the study design, performed the statistical analysis and drafted the article.

Abstract

Background

Nucleoside reverse transcriptase inhibitors (NRTIs) are often administered in salvage therapy even if genotypic resistance tests (GRTs) indicate high-level resistance, but little is known about the benefit of these additional NRTIs.

Methods

The effect of <2 compared to 2 NRTIs on viral suppression (HIV-1 RNA <50 copies/mL) at week 24 was studied in salvage patients receiving raltegravir. Intent-to-treat and per-protocol analyses were performed; last observation carried forward imputation was used to deal with missing information. Logistic regressions were weighted to create a pseudo-population in which the probability of receiving <2 and 2 NRTIs was unrelated to baseline factors predicting treatment response.

Results

One-hundred thirty patients were included, of whom 58.5% (n=76) received <2 NRTIs. NRTIs were often replaced by other drug classes. Patients with 2 NRTIs received less additional drug classes compared to patients with <2 NRTIs [median (IQR): 1 (1-2) compared with 2 (1-2), *P* Wilcoxon <0.001]. The activity of non-NRTI treatment components was lower in the 2 NRTIs group compared with the <2 NRTIs group [median (IQR) genotypic sensitivity score: 2 (1.5-2.5) compared with 2.5 (2-3), *P* Wilcoxon<0.001]. The administration of <2 NRTIs was associated with a worse viral suppression rate at week 24. The odds ratios were 0.34 (95% confidence interval: 0.13 to 0.89, *P*=0.027) and 0.19 (95% confidence interval: 0.05 to 0.79, *P*=0.023) when performing the last observation carried forward and the per-protocol approach, respectively.

Conclusion

Our findings showed that partially active or inactive NRTIs contribute to treatment response, and thus the use of 2 NRTIs in salvage regimens that include raltegravir seems warranted.

Introduction

The treatment options for patients infected with highly drug-resistant HIV markedly improved with the introduction of new antiretroviral compounds, such as fusion inhibitors, second-generation nonnucleoside reverse transcriptase inhibitors (NNRTIs), or new boosted protease inhibitors (PIs), CCR5 antagonists, and integrase inhibitors.[1-7] To date, knowledge about the optimal combination of these compounds in salvage therapy is lacking. Nucleoside reverse transcriptase inhibitors (NRTIs) are often co-administered in salvage therapy, even if genotypic resistance tests (GRTs) indicate high-level resistance. A therapeutic benefit is assumed because of the possible residual activity of these NRTIs and the maintenance of a resistant virus with reduced replicative capacity.[8-12] On the other hand, costs, drug-drug interactions, tolerability and toxicity of these additional NRTIs have to be taken into account. NRTIs can cause mitochondrial dysfunction by inhibiting the DNA γ-polymerase resulting in plasma hyperlactataemia and variable clinical syndromes, such as lipoathrophy and peripheral neuropathy.[13-21]

The clinical benefit of NRTIs with decreased activity due to drug resistance mutations to date has not been properly assessed. The number of antiretroviral compounds has increased, and additional drug classes have become available, making NRTIs potentially expendable in salvage therapy.

Here, we focused on salvage regimens including raltegravir (RAL) because this drug is now frequently used in Switzerland to treat patients with highly resistant viruses.[22] Using data from the highly representative Swiss HIV Cohort Study (SHCS),[23, 24] we report on the genotypic activity and composition of salvage therapies with RAL and the effect of partially active or inactive NRTIs on the viral suppression rate.

Methods

Data and patient selection

Data from the SHCS were included for our analysis (up to June 30, 2010). The SHCS is a nationwide, clinic-based cohort study with continuous enrolment and at least semi-annual study visits (www.shcs.ch).[24] It has been approved by ethical committees of all participating institutions, and written informed consent has been obtained from participants. The SHCS drug resistance database contains all HIV resistance tests performed by the 4 authorized laboratories in Switzerland using commercial assays (Viroseq Vs.1 PE Biosystems, Rotkreuz, Switzerland; Virsoseq

Vs. 2, Abbott AG, Baar, Switzerland; vircoTYPE HIV-1 Assay, Virco Lab, Mechelen, Belgium) and in-house methods.[25] Sequences are stored in SmartGene's (Zug, Switzerland) Integrated Database Network System (IDNS version 3.5.8).[26]

Study population

To analyze the effect of partially active or inactive NRTIs in salvage therapy, the SHCS was screened for patients who started a regimen containing RAL. Inclusion criteria were GRT on antiretroviral therapy (ART) prior to the RAL start and baseline HIV-1 RNA >500 copies/mL. Patients receiving more than 2 NRTIs were excluded from the study due to the small number of cases (n=12). For further analyses, patients receiving 0 or 1 NRTI were considered as one group and compared to patients receiving 2 NRTIs beside RAL. This classification turned out to be appropriate because patients treated with 0 and 1 NRTI had similar characteristics, and results did not differ markedly when analyzing these two groups separately (not shown).

Baseline characteristics and estimated activity of available treatment options

Patient characteristics were compared between patients receiving <2 NRTIs and 2 NRTIs with Fisher's exact test (categorical variables) and Wilcoxon rank-sum test (continuous variables). The baseline was set at the date of RAL start. The self-reported adherence was categorized in 2 groups: patients who never missed a drug and patients who missed ≥1 drug in the 4 weeks preceding the study visit.[27] To assess the availability of active antiretroviral compounds, results from Stanford interpretation algorithm (version 6.0.8) were mapped to a genotypic sensitivity score (GSS) for all approved drugs except enfuvirtide (T20), maraviroc (MAR), and RAL. The 5 resistance categories from the Stanford algorithm were regrouped as follows: viruses with a GSS less than 15 were considered as fully susceptible (GSS=1), those with a GSS between 15 and 59 were considered to have intermediate resistance (GSS=0.5), and those with a GSS greater than 59 were considered to be fully resistant (GSS=0). If T20 and MAR have not previously been included in a failing regimen, they were considered fully susceptible because transmission of HIV with resistance to T20 is very rare and coreceptor tropism testing was always performed prior to MAR prescription (Trofile assay, Monogram Biosciences, San Francisco, CA).[28]

Virological outcome

The effect of NRTIs in salvage therapies with RAL was assessed at week 24. The viral suppression rate (HIV-1 RNA <50 copies/mL) was analyzed and different approaches were implemented as follows: an intent-to-treat analysis was performed with two different methods dealing with missing information, last observation carried forward (LOCF) and missing equal failure (m = f), and a per-protocol analysis. For the per-protocol analysis, only patients who did not change, stop, or interrupt treatment until week 24 and who had a viral load measurement between week 18 and week 30 were included.

Logistic regressions were performed and adjusted for ethnicity, age, sex, the GSS of the treatment (without NRTIs), number of drug classes, HIV-1 RNA and CD4 cell count before RAL treatment start. In the present study, confounding by indication must be addressed, because many factors, for example, number of drug classes in the background regimen, GSS of available drugs, or adherence, may influence not only the suppression rate, but also the number of NRTIs physicians chose for the salvage therapy. A solution to overcome a selection bias is to perform a marginal structural model.[29] Weights were defined as the inverse of the probability for receiving <2 NRTIs as estimated by multivariable logistic regression including the following possible confounders: sex, adherence, age, transmission category, ethnicity, MAR, etravirine (ETV), or darunavir (DRV) in the background treatment, GSS of available NRTIs, GSS of PIs and NNRTIs in the salvage therapy, CD4 nadir, baseline HIV-1 RNA, year of treatment, and whether the patient was ever treated with mono/dual NRTI therapy. This method creates a pseudo-population, in which the probability for receiving <2 or 2 NRTIs is unrelated to baseline factors which are also prognostic for the treatment response (Appendix 1). Multicollinearity was checked and a variance inflation factor (VIF) <3 was tolerated for regression models. To check whether single observations had a disproportionately large impact on our results due to the weighting, the analysis was repeated 1000 times on bootstrapped data sets.

To confirm results, an additional analysis was performed assessing time to viral suppression with a Cox regression model. The same covariables were included as in the logistic regression described above and the same procedure was followed to calculate the weights. Patients were included when they had at least 1 HIV-1 RNA measured and they were censored when they changed, stopped, or interrupted therapy.

Statistical analyses were performed with Stata 11 SE (StataCorp, College Station, TX), all confidence intervals (CI) are 95% CI and the level of significance was set at *P*=0.05.

Results

Study population and baseline characteristics

A total of 142 patients who had a viral load >500 copies/mL, a GRT performed before RAL treatment start and follow-up HIV-1 RNA measurements were considered for analysis. Patients who received more than 2 NRTIs were excluded from further analysis (11 with 3 NRTIs, 1 with 4 NRTIs). Patients who received no NRTI (n=38, 26.8%) or 1 NRTI (n=38. 26.8%) were handled as one group and compared to patients receiving 2 NRTIs (n=54, 38.0%).

Most baseline characteristics were similar between patients with <2 NRTIs and 2 NRTIs (Table 1), but patients with 2 NRTIs were younger, had more often baseline HIV-1 RNA >100,000 copies/mL and tended to have started the first ART later. The self-reported adherence during the 4 weeks proceeding the study visit before RAL start was similar: 72.2% (2 NRTIs group) and 71.1% (<2 NRTIs group). Additional factors that might be a sign of non-adherence were tested as follows: the number of therapies patients started, the number of treatment interruptions (cessation of ART and resumption at a later date), psychiatric treatment in the past, alcohol abuse, current intravenous drug use or smoking. All these characteristics were similar among groups (data not shown).

The median number of NRTI, NNRTI and PI mutations (International AIDS Society [IAS-USA] drug resistance mutations printed in bold)[30] was comparable between patients with <2 and 2 NRTIs, 9 [interquartile range (IQR): 5-13] and 10 (7-14.5) (*P*=0.100), respectively. Of note, the median number of NRTI mutations was lower in the 2 NRTIs group, 4 (1-5) compared to 5 (3-5) in the <2 NRTIs group (*P*=0.040). The number of major PI and NNRTI mutations was not significantly different.

Tabelle 1. Baseline characteristics of patients who started salvage treatment with raltegravir

	<2 NRTIs (n=76)*	2 NRTIs (n=54)*	*P**
Socio-demographic factors			
Median (IQR) age (in yrs)	49 (42.5-51)	43 (40-48)	0.009
Sex			
female	28.9% (19.1-40.5)	25.9% (15.0-39.6)	0.704
male	71.0% (59.5-80.9)	74.1% (60.4-85.0)	
Ethnicity			
white	82.9% (72.5-90.6)	87.0% (75.1-94.6)	0.519
other	17.1% (9.4-27.5)	13.0% (5.4-24.9)	
Risk group			
MSM	51.3% (39.6-63.0)	55.6% (41.4-69.1)	0.972
HET	28.9% (19.1-40.5)	25.9% (15.0-39.6)	
IDU	15.8% (8.4-26.0)	14.8% (6.6-27.1)	
other	4.0% (0.8-11.1)	3.7% (0.5-12.8)	
Immunological and virological factors			
Baseline HIV-1 RNA (copies/mL)			
500-9,999	43.4% (32.1-55.3)	37.0% (24.3-51.3)	0.068
10,000-99,999	44.7% (33.3-56.6)	35.2% (22.7-49.4)	
≥100,000	11.8% (5.6-21.3)	27.8% (16.5-41.6)	
Median (IQR) CD4 (cells/μL)	226 (128.5-302.5)	256 (94-314)	0.962
Median (IQR) CD4 nadir (cells/μL)	71.5 (18.5-176)	82 (33-172)	0.498
Subtype			
B	80.3% (69.5-88.5)	87.0% (75.1-94.6)	0.310
other	19.7% (11.5-30.5)	13.0% (5.4-24.9)	
CDC stage			
A	23.7% (14.7-34.8)	18.5% (9.3-31.4)	0.702
B	38.2% (27.3-50.0)	44.4% (30.9-58.6)	
C	38.2% (27.3-50.0)	37.0% (24.3-51.3)	
Treatment history			
Median (IQR) year of therapy start	1995 (1993-1996)	1996 (1994-1998)	0.037
Ever mono/dual NRTI therapy	88.2% (78.7-94.4)	79.6% (66.5-89.4)	0.184
Prior DRV	2.6% (0.3-9.2)	9.3% (3.1-20.3)	0.099
Prior ETV	5.3% (1.5-12.9)	3.7% (0.5-12.8)	0.676
Prior MAR	2.6% (0.3-9.2)	0.0% (0.0-6.6)	0.230
Prior T20	18.4% (10.4-29.0)	18.5% (9.3-31.4)	0.989

*Percentage (95% confidence interval) or median (IQR), *P* value based on Fisher's exact test (categorical variables) or Wilcoxon rank-sum test.
MSM, men who have sex with men.

Available treatment options based on genotypic data

Some treatment options differed between patients with 2 and <2 NRTIs and were important reasons to perform a weighted logistic regression. The GSS of the best two NRTIs was <1, 1 and >1 in 29.6%, 20.4% and 50.0% of patients with 2 NRTIs, slightly higher than in the <2 NRTIs group (43.4%, 26.3% and 30.3%, *P*=0.075). As shown in Table 2, the NRTI with the highest estimated activity was tenofovir (TDF), with only 3.7% (2 NRTIs group) and 11.8% (<2 NRTIs group) being fully resistant. In contrast, full resistance against the following NRTI groups was common in patients with 2 and <2 NRTIs: zidovudine/stavudine (37.0% and 59.2%), emtricitabine (FTC)/lamivudine (3TC) (81.5 and 85.5%) and abacavir (ABC)/didanosine (DDI)

(37.0% and 52.6%). Resistance against the new NNRTI ETV was rare 1.9% (2 NRTIs group) and 5.3% (<2 NRTI group). In contrast, full resistance against first line NNRTIs (EFV and NVP) was common in patients with 2 and <2 NRTIs, 63.0% and 68.4%, respectively. DRV was the best PI with an estimated full activity in 73.9% of the cases, followed by tipranavir (48.5%), lopinavir (43.9%), saquinavir (39.2%), and indinavir (38.5%).

Table 2. Genotypic activity of potential antiretroviral compounds for salvage treatment

Estimated activity*	<2 NRTIs	2 NRTIs	*P*
GSS of NRTIs			
ZDV/D4T			
1	23.7% (14.7-34.8)	33.3% (21.1-47.5)	0.041
0.5	17.1% (9.4-27.5)	29.6% (18.0-43.6)	
0	59.2% (47.3-70.3)	37.0% (24.3-51.3)	
FTC/3TC			
1	6.6% (2.2-14.7)	14.8% (6.6-27.1)	0.212
0.5	7.9% (3.0-16.4)	3.7% (0.5-12.8)	
0	85.5% (75.6-92.5)	81.5% (68.6-90.8)	
ABC/DDI			
1	9.2% (3.8-18.1)	35.2% (22.7-49.4)	0.001
0.5	38.2% (27.3-50.0)	27.8% (16.5-41.6)	
0	52.6% (40.8-64.2)	37.0% (24.3-51.3)	
TDF			
1	18.4% (10.4-29.0)	42.6% (29.2-56.8)	0.006
0.5	69.7% (58.1-79.8)	53.7% (39.6-67.4)	
0	11.8% (5.6-21.3)	3.7% (0.5-12.8)	
GSS of NNRTIs			
EFV/NVP			
1	28.9% (19.1-40.5)	35.2% (22.7-49.4)	0.735
0.5	2.6% (0.3-9.2)	1.9% (0.1-9.9)	
0	68.4% (56.8-78.6)	63.0% (48.7-75.7)	
ETV			
1	50.0% (38.3-61.7)	61.1% (46.9-74.1)	0.347
0.5	44.7% (33.3-56.6)	37.0% (24.3-51.3)	
0	5.3% (1.5-12.9)	1.9% (0.1-9.9)	
GSS of PIs			
Highest scoring PI			
1	73.7% (62.3-83.1)	79.6% (66.5-89.4)	0.423
0.5	23.7% (14.7-34.8)	20.4% (10.6-33.5)	
0	2.6% (0.3-9.2)	0.0% (0.0-6.6)	

*A GSS of 1 denotes full susceptibility, 0.5 intermediate resistance and 0 full resistance. GSS was calculated with Stanford algorithm version 6.0.8.
D4T, stavudine; ZDV, zidovudine

Composition of the salvage therapy with RAL

The composition of the non-NRTI treatment patients received together with RAL differed markedly between groups. The number of non-NRTI drugs beside RAL was lower in the 2 NRTIs group. The percentage of patients with ≤1, 2 and 3 drugs was

68.5%, 27.8% and 3.7% compared to 30.3%, 56.6% and 13.2% (*P*<0.001). Also the number of drug classes beside NRTIs and RAL was lower in the 2 NRTIs group [median IQR: 1 (1-2) compared with the <2 NRTI group: median (IQR): 2 (1-2), *P* Wilcoxon<0.001).

As shown in Figure 1, most patients with 2 and <2 NRTIs additionally received a boosted PI, 66.7% and 80.3% (*P*=0.102). Most patients with a boosted PI received DRV (74.3%). NNRTIs also were often co-administered, in 44.4% (2 NRTIs group) and 68.4% (<2 NRTIs group) of cases (*P*=0.007). Patients with a NNRTI most often had ETV (88.2%). MAR and T20 were rarely administered (3.7% and 18.4%, *P*=0.014; 3.7% and 6.6%, *P*=0.699).

In the 2 NRTIs group, the predominant NRTI combination was 3TC/FTC and TDF (38/54, 70.4%), followed by ABC and 3TC (14.8%), ABC and TDF (11.1%), TDF and DDI, and 3TC and zidovudine (each 1.9%). Patients with 1 NRTI most often received 3TC (16/38, 42.1%) or TDF (15/38, 39.5%). Three patients received ABC (7.9%), 2 DDI (5.3%) and 1 each zidovudine and stavudine (2.6%).

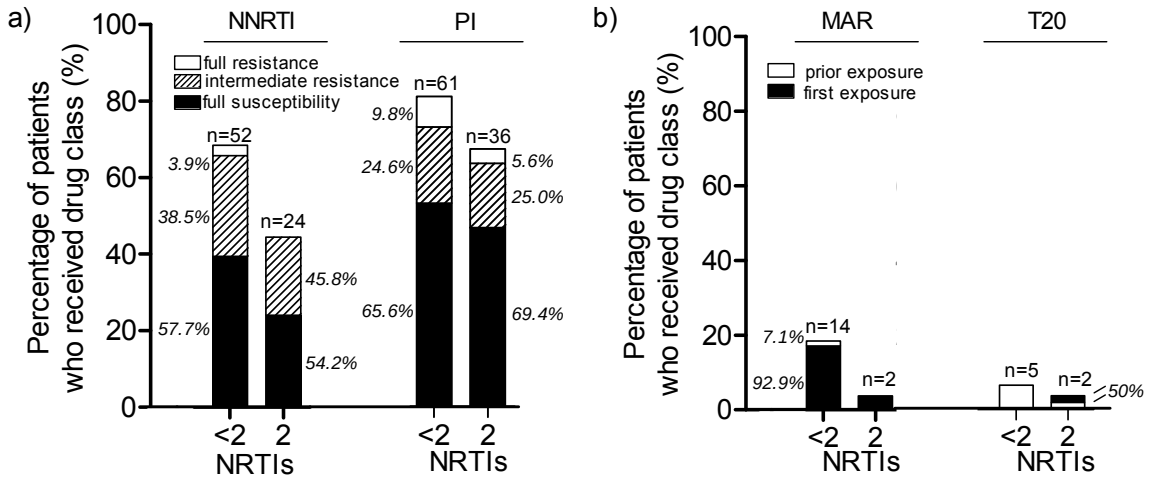

Figure 1. Background regimen of patients who receive <2 and 2 NRTI in addition to RAL. A, Percentage of patients who received NNRTI or PI, the proportions of drugs of full resistance, intermediate resistance, or full susceptibility are indicated in italics. B, Percentage of patients who received MAR or T20, the proportions of prior exposure are indicated in italics.

Estimated genotypic activity of the salvage therapy

The GSS of all non-NRTI drugs in the salvage therapy was lower in the 2 NRTIs group with a median GSS of 2 (1.5-2.5) compared with 2.5 (2-3) (*P*<0.001). However, when also considering the GSS of NRTIs the overall GSS of the treatment tended to be higher in the 2 NRTIs group 3 (2.5-3.0) compared with 2.5 (2-3, *P*=0.059).

The contribution to the GSS of each NRTI was similar in the <2 NRTIs and the 2 NRTIs group, the GSS was <0.5, 0.5 and >0.5 in 46.3%, 31.5% and 22.2% compared with 47.4%, 31.6% and 21.1% (*P*=1.000) cases. Most patients (47/63, 74.6%) receiving 3TC/FTC had viral strains carrying the M184I/V mutations, in the <2 and 2 NRTIs group 81.3% and 76.6%, respectively.

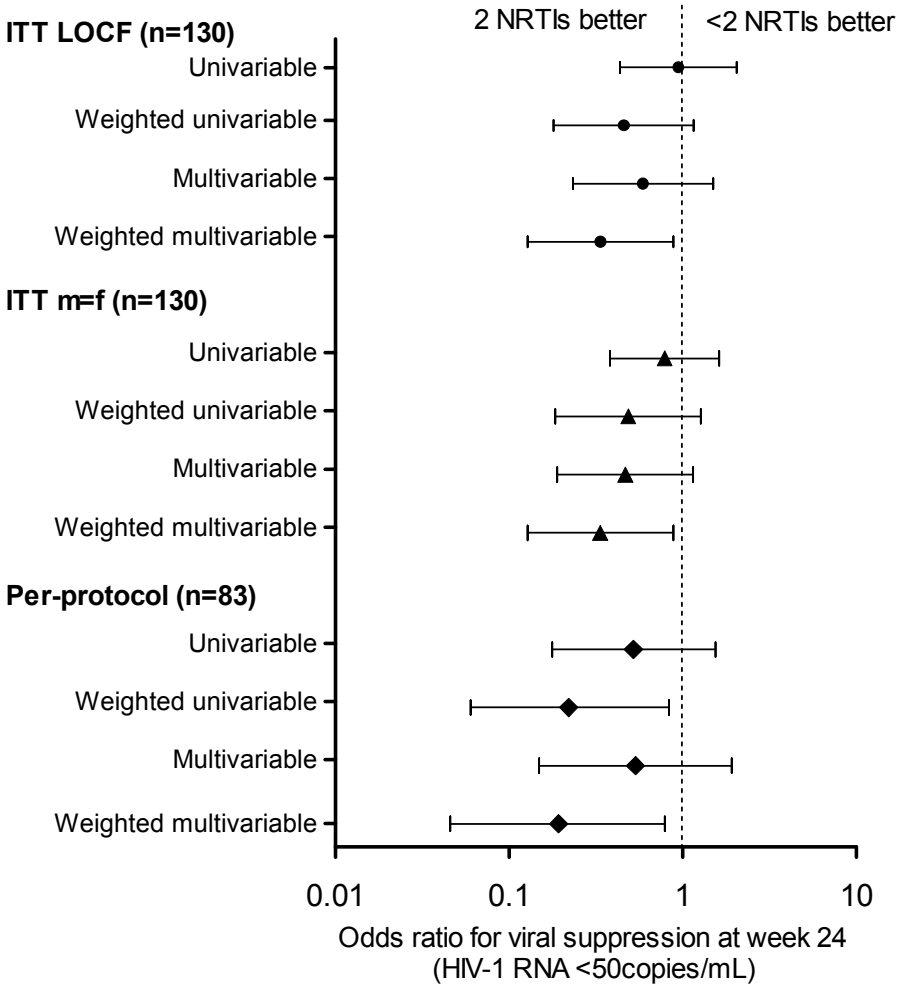

Figure 2: Logistic regression was performed to compare the viral suppression rate at week 24 between patients treated with <2 NRTIs or 2 NRTIs in a salvage treatment with RAL. Different approaches were compared: intent-to-treat analysis with missing equal failure (ITT m=f), ITT LOCF and a per-protocol analysis.

Virological outcome

The described differences in salvage therapy composition and in particular the higher number of drug classes included in the <2 NRTIs group are possibly interfering with our aim to measure the effect of partially active or inactive NRTIs. For this purpose a

marginal structural model was performed. The model creates a “pseudo-population” in which group differences in salvage treatment composition are balanced.

With the LOCF approach, the crude percentages of patients who achieved viral suppression at week 24 were 72.2% and 71.0% for patients with 2 and <2 NRTIs, respectively. The median (IQR) week of measurement was 24 (20-27) and similar between patients with 2 and <2 NRTIs [24.1 (19.9-26.6) compared with 23.9 (20.1-27.5)]. About 2.6% (2 NRTIs group) and 5.6% (<2 NRTIs group) had no RNA measurement performed. A similar number of patients stopped, interrupted, or changed treatment before week 24, 22.2% (12 of 54) and 29.0% (22 of 76) in the 2 and <2 NRTIs group, respectively (*P*=0.425). Toxicity was the reason for the change among 16.7% (2 of 12) and 36.4% (8 of 22) of the cases. (*P*=0.430). As shown in Figure 2, multivariable logistic regressions showed that patients treated with <2 NRTIs compared with 2 NRTIs had a decreased chance to achieve viral suppression [multivariable odds ratio (OR): 0.59, *P*=0.269; weighted multivariable OR: 0.34, *P*=0.027] (table 3). The robustness of the results was tested with a bootstrap analysis (1000 replications), it yielded a similar result [mean multivariable weighted OR: 0.41 (fifth and 95th percentiles: 0.11-0.98)], suggesting that the observed differences were not hinging on a few specific observations in our dataset, but were broadly consistent.

The crude percentage of patients with suppressed viral load was slightly lower when using the m = f approach (2 NRTIs: 64.8%, <2 NRTIs: 59.2%). The beneficial effect of 2 NRTIs was confirmed in multivariable (OR: 0.47, *P*=0.099), and in weighted multivariable models (OR: 0.33, *P*=0.027) (Fig. 2). Also the per-protocol analysis confirmed results. Eighty-three patients were included who did not change, stop, or interrupt treatment until week 24 and who had a viral load measurement performed within the given time frame (multivariable OR: 0.54, *P*=0.337, weighted multivariable OR: 0.19, *P*=0.023).

Different sensitivity analyses were performed to verify the results. Because the higher GSS of NRTIs in the 2 NRTIs group might partially explain the results, a sub-analysis including only patients with a cumulative NRTI GSS ≤0.5 in the regimen was performed (n=93 ITT LOCF, n=60 per-protocol). It was confirmed that additional NRTIs with low activity are beneficial for virological outcome (ITT LOCF: weighted multivariable OR: 0.13, 95% CI: 0.03-0.55; per-protocol: OR: 0.06, 95% CI: 0.01-0.38). Because the last GRT was not always performed immediately before RAL

start, the estimation of the GSS might be imprecise. Therefore, a logistic regressions including exclusively patients who had a GRT on the last failing regimen was performed [ITT LOCF (n=104): weighted multivariable OR: 0.36, 95% CI: 0.11-1.18, per-protocol (n=65): OR: 0.07, 95% CI: 0.01-0.46].

As an additional analysis time to viral suppression was studied. Patients were included if they had at least 1 RNA measurement performed before treatment change, stop, or interruption (n=109). The frequency of RNA measurements after RAL start was similar between groups (data not shown). Compared with patients with 2 NRTIs, patients with <2 NRTIs had a longer time to viral suppression. The hazard ratio with the multivariable and weighted multivariable regression was 0.63 (95% CI: 0.39-1.03, *P*=0.064) and 0.54 (95% 0.37-0.80, *P*=0.002), respectively.

Table 3. Weighted multivariable logistic regression analyzing virological suppression at week 24.

Characteristics	**HIV-1 RNA<50 copies/mL (n=93)**	**HIV-1 RNA ≥50 copies/mL (n=37)**	**Weighted multivariable OR (95% CI)**	***P***
Number of NRTIs				
2	39 (72.2%)	15 (27.8%)	1 (ref)	
<2	54 (71.0%)	22 (28.9%)	0.34 (0.13 to 0.89)	0.027
Sex				
Male	66 (70.2%)	28 (29.8%)	1 (ref)	
Female	27 (75.0%)	9 (25.0%)	2.19 (0.57 to 8.45)	0.254
Ethnicity				
White	79 (71.8%)	31 (28.2%)	1 (ref)	
Other	14 (70.0%)	6 (30.0%)	1.10 (0.30 to 4.01)	0.887
Risk				
IDU	14 (70.0%)	6 (30.0%)	1.38 (0.41 to 4.65)	0.608
Other	79 (71.8%)	31 (28.2%)	1 (ref)	
Median (IQR) age (in yrs)	48 (41-51)	44 (41-48)	1.10 (1.03 to 1.17)	0.002
Median (IQR) HIV-1 RNA (log_{10} copies/mL)	4.2 (3.3-4.7)	4.7 (3.8-5.1)	0.51 (0.29 to 0.87)	0.015
CD4 count (cells/µL)				
<200	39 (69.6%)	17 (30.4%)	1 (ref)	
≥200	54 (73.0%)	20 (27.0%)	0.91 (0.36 to 2.32)	0.850
GSS of the treatment (without NRTIs)				
0-1.5	14 (56.0%)	11 (44.0%)	1 (ref)	
2-2.5	54 (77.1%)	16 (22.9%)	3.17 (0.83 to 12.1)	0.092
≥3	25 (71.4%)	10 (28.6%)	4.04 (0.55 to 29.9)	0.172
Median (IQR) drug classes	3 (2-3)	3 (2-3)	1.17 (0.50 to 2.74)	0.711

MSM, men who have sex with men

Discussion

The availability of second-line antiretroviral agents and the introduction of new drug classes increased the options for salvage treatment markedly and raised the question of the optimal combination of compounds. Particularly, the role of genotypic partially

or completely inactive NRTIs in such situations is unknown. In our study, we saw that NRTIs were often replaced by other drug classes, such as second-line NNRTIs, boosted PIs, T20 or MAR. Importantly, patients who received two inactive or partially active NRTIs were more likely to achieve viral suppression at week 24. These NRTIs might have a residual antiretroviral activity or select viruses with reduced replicative capacity which might be favourable to achieve viral suppression.[9] The presence of 2 NRTIs increased the chance to achieve viral suppression three times. Single NRTIs did not significantly increase the chance to achieve viral suppression but tended to show an additional benefit compared to NRTI-sparing regimen (data not shown). Also the time to viral suppression was faster when 2 NRTIs were given. A short time to suppression might be beneficial because it may decrease the chance to accumulate resistance associated mutations in the very early phase of therapy. These finding were consistent and were confirmed with different approaches and sensitivity analyses.

The use of NRTIs in salvage therapies has several potential advantages. In contrast to new compounds, NRTIs are well studied after 20 years of use: Their long-term toxicities are well characterized and the potential for drug-drug interactions is low. Costs are much lower compared with newer antiretroviral compounds, which are particularly relevant for developing countries and will become more important in the future when generic antiretroviral agents will be available.

Previous studies showed that NRTI-sparing regimens suppress viremia in treatment-naïve and treatment-experienced patients but increase the probability to select for drug resistance mutations. They reduce the frequency of lipoatrophy, but other adverse events occurred when combining remaining drug classes (eg, PIs and NNRTIs.[31-35]

Preliminary results from another study addressing the effect of inactive NRTIs in salvage therapy are in contradiction with our findings. However, in contrast to our study, no adherence data were available, no weighting was performed, the number of patients with <2 NRTIs was very small (27 compared to 76 in our study) and it was not differentiated between patients receiving salvage treatment with RAL, MAR or ETV.[36]

We used marginal structural models to overcome confounding by indication. The model performed in this study simulated a hypothetical randomized controlled trial in which patients were randomly assigned to receive a treatment with <2 or 2 NRTIs. As

in any observational studies, it is impossible to exclude unmeasured confounding. In particular, we cannot fully exclude that there were additional factors, which led physicians to choose a treatment with <2 NRTIs, but which were also associated with a worse treatment outcome. However, in absence of a randomized controlled trial, observational studies represent the best available evidence. To analyze long-term effects and NRTI-related toxicities large cohort collaborations will be needed.

The possibility to maintain NRTI in the salvage regimen despite the presence of major drug resistance mutation is of high relevance because the drug pipeline of new antiretroviral agents starts to decline and on a global scale resistance will continue to accumulate. [37-40]

To summarize, our study demonstrated that partially active or inactive NRTIs showed a beneficial effect on the short-term virological outcome in patients receiving RAL. Therefore, our study supports the strategy to administer two NRTIs in salvage therapy with RAL even if inactive or only partially active according to GSS. The negative impact on viral fitness by maintaining drug resistance mutations and the residual activity of NRTIs must not be underestimated. However, the benefit of these NRTIs should be balanced with potential complications because complex antiretroviral regimens can be associated with increased toxicity or poor adherence. Further studies and collaborations are needed to support our findings and to analyze the long-term benefit of partially active or inactive NRTIs.

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(Head of Data Center), Rudin C (Chairman of the Mother & Child Substudy), Schmid P,Schultze D, Schüpbach J, Speck R, de Tejada BM, Taffé P, Telenti A, Trkola A, Vernazza P, Weber R, Yerly S.

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Appendix 1*

The standard approach to study the impact of partially active or inactive NRTIs on viral suppression is a logistic regression model, but Robins[41] showed that this approach may be biased when there is a (1) time-dependent covariate that is a predictor for both, the event of interest and the exposure, and (2) past treatment history predicts the level of the covariate. In our study, the GSS of available non-NRTIs is such an example, patients with a high GSS of non-NRTIs are more likely to achieve viral suppression (event of interest) and patients with a low GSS of non-NRTIs tended to receive more NRTIs (exposure), and the level of the GSS of non-NRTIs is influenced by the treatment history. The marginal structural model (MSM) is one possibility to deal with this problem. The parameters of the MSM can be estimated with using inverse-probability-of-treatment weights. The crude logistic regression is modified by weighting each subject i by ω_i. For example, if a given patient has a weight of $\omega_i=5$, the patient contributes five copies of him- or herself to the pseudo-population. Thus, the model simulates a randomized controlled trial in which patients were randomly assigned to receive a treatment with <2 or 2 NRTIs.[29, 41, 42]

On the basis of the study of Fewell et al,[43] we performed the following analysis in Stata to estimate the MSM:

The weights were derived using a logistic regression model:

```
xi: logit number_nrti sex adherence age i.transmission ethnicity background_maraviroc ///
background_etravirine background_darunavir background_t20 gss_nrti gss_nnrti_pi ///
cd4_nadir log_bl_rna i.bl_cd4 year_of_treatment i.monodual_nrti, or
```

Probability of receiving 0 or 1 NRTIs:

```
predict pw if e(sample)
```

Probability of the treatment patients actually got:

```
replace pw = (1-pw) if number_nrti==0
```

Inverse probability of treatment weights:

```
gen ipw= 1/pw
```

Examination of the distribution of the weights:

```
summ ipw,detail
```

Marginal structural model:

```
xi: logistic supression_week24 i.number_nrti ethnicity sex age i.transmission ///
i.gss_of_no_nrtis log_bl_rna i.bl_cd4 number_of_drug_classes [pweight=ipw]
```

* Appendix 1 is not included in the published version of the manuscript. The journal did not provide enough space to add it. However, in the framework of the thesis it is valuable to explain the used methods in more detail.

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Chapter 4

Emergence of multi-nucleoside resistance

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Predictors for the emergence of the 2 multi-nucleoside/nucleotide resistance mutations 69 insertion and Q151M and their impact on clinical outcome in the Swiss HIV Cohort Study (SHCS)

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Abstract

The 69 insertion and Q151M mutations are multi-nucleoside/nucleotide resistance mutations (MNR). The prevalence among 4078 antiretroviral (ART)-experienced individuals was <1.3%. Combined ART fully prevented MNR in subtype B infections. Case-control studies were performed to identify risk factors. Control subjects were patients with ≥3 thymidine-analogue mutations. The 69 insertion study (27 control subjects, 14 case patients) identified didanosine exposure as a risk (odds ratio, 5.0 per year, *P*=0.019), whereas the Q151M study (which included 44 control subjects and 25 case patients) detected no associations. Following detection, individuals with Q151M tended to have lower suppression rates and higher mortality rates, relative to control subjects. Additional studies are needed to verify these findings in non-subtype B infections.

Introduction

The introduction of highly-active antiretroviral therapy (HAART) reduced morbidity and mortality of human immunodeficiency virus (HIV) infected patients. However, drug resistance continues to emerge in association with treatment failure.[1]

The 69 insertion and Q151M mutation are multi-nucleoside/nucleotide resistance (MNR) mutations on the reverse transcriptase that affect the activity of all approved nucleoside reverse transcriptase inhibitors (NRTIs). While 69 insertion confers full resistance to all drugs, tenofovir retains some activity when Q151M is present.[2-4] MNR mutations occur rarely in European settings (prevalence <1% to 3.6%). The prevalence in resource-limited countries has not been analyzed, but recent reports indicate that it has increased.[5-8] The possible increase in MNR HIV strains is of great concern because of the very limited options for salvage treatment in resource-limited settings and the general lack of understanding as to how to optimally treat patients who have MNR infections.[9]

We aimed to identify predictors for the emergence of the 69 insertion and Q151M mutation in the Swiss HIV Cohort Study (SHCS) and studied outcomes of salvage regimens applied for treatment of MNR HIV infections.

Methods

Data and patient selection

Our analysis included data from the SHCS and the SHCS drug resistance database up to February 2010.[10, 11] The SHCS has been approved by ethical committees of all participating institutions, and written informed consent has been obtained from participants.

Prevalence of acquired and transmitted MNR mutations

The prevalence of the 69 insertion and the Q151M mutation was analyzed among treatment-experienced (at least 30 days exposure) and treatment-naïve patients. Among treatment-naïve patients, possible transmission clusters were identified through phylogenetic methods. These analyses were performed with PHYLIP 3.6 (distributed by J. Felsenstein), using the F84 nucleotide substitution model and the neighbor-joining tree algorithm with 1000 bootstraps. To avoid interference of treatment history, all major International AIDS Society-USA drug resistance-associated amino acid positions were deleted from the sequences prior to analysis.[12]

Case-control study to determine predictors for MNR

We compared patients with MNR detected with patients who carried viruses with ≥3 thymidine analogue mutations (TAMs), either from the TAM 1 (M41L, L210W, T215Y) or the TAM 2 pathway (D67N, K70R, T215F, K219K/E). The rationale was to establish a control group consisting of highly NRTI-experienced individuals with comparable characteristics, except for the occurrence of 69 insertion or Q151M. Separate matched case-control studies were performed for each of the 2 MNR mutations. Control patients were matched 2:1 on the basis of the fist antiretroviral treatment (ART) received and the time between ART initiation and the detection of MNR mutations (for case patients) or ≥3 TAMs (for control subjects). Inclusion was restricted to individuals infected with HIV subtype B who started ART with single-class NRTI therapy.

Conditional logistic regression analyses were performed to identify risk factors for the emergence of 69 insertion and Q151M. Variables tested included the time spent on specific NRTIs and adjustments for the following potential confounders: sex, age, ethnicity, risk group, HIV-1 RNA level, and CD4+ cell count at time of detection of MNR mutations (for case patients) or ≥3 TAMs (for control subjects).

Factors associated with attaining undetectable viral loads after detection of MNR mutations

Virological outcomes after detection of MNR were analyzed for patients from the case-control studies with >1 follow-up HIV-1 RNA measurement. Characteristics and treatments were compared between patients who ever achieved 2 consecutive undetectable HIV-1 RNA levels <50 copies/mL and patients who did not. Fisher's exact test (categorical) and Wilcoxon rank-sum test (continuous variables) were used.

Association of all-cause mortality with detection of MNR mutations

Cox proportional hazard models for matched case-control data were estimated to analyze the time to all-cause mortality after detection of MNR mutations (case patients) or ≥3 TAMs (control subjects). Models were stratified by years of detection of MNR mutations or ≥3 TAMs (1998, 1999-2003, after 2003). The proportional hazard assumption was verified by analyzing Schoenfeld residuals.

Statistical analyses were performed with Stata 11 SE (StataCorp), all confidence intervals (CI) are 95% CIs, and the level of significance was set at *P*=0.05.

Results

Prevalence of MNR in treatment-experienced patients

The SHCS included 19 (0.5%) of 4078 and 34 (0.8%) of 4078 treatment-experienced patients who carried viruses with the 69 insertion and Q151M mutation, respectively. Most patients in the 69 insertion and Q151M group were treated with mono- or dual NRTI therapy (14 [73.7%] of 19 and 30 [88.2%] of 34), respectively. MNR was never detected in patients who were exclusively treated with HAART (2 NRTIs and 1 boosted protease inhibitor [PI]/nonnucleoside reverse transcriptase inhibitor [NNRTI]). The median duration of ART until detection was 6.8 for the 69 insertion group and 5.5 years for the Q151M group. The median years of detection were 2000.5 (range: 1995-2007) and 2000 (range: 1995-2006), respectively. Most individuals who carried viruses with MNR were infected with subtype B viruses (18 [94.7%] of 19 and 29 [85.3%] of 34, respectively). All additional analyses were restricted to this subtype.

Evidence for MNR transmission among treatment-naïve patients

We screened 5692 sequences from treatment-naïve patients and detected the 69 insertion 3 times (0.05%) and Q151M once (0.02%). The phylogenetic analysis provided strong evidence of forward transmission of the 69 insertion from 1 index to 2 patients (100% bootstrap support). All these patients were men who had sex with men from the same study center. For Q151M, no transmission cluster was detected.

Predictors for the emergence of MNR in patients infected with HIV subtype B

For the 69 insertion case-control study, matching criteria fitted for 14 case patients (69 insertion) and 27 control subjects (≥3 TAMs). Interestingly, years spent receiving didanosine were significantly associated with the emergence of 69 insertion in univariable (odds ratio [OR], 3.4; 95% CI, 1.2-9.6; *P*=0.019) and multivariable models (OR, 5.0; 95% CI, 1.3-19.3; *P*=0.019).

For the Q151M case-control study, 25 case patients and 44 control subjects (≥3 TAMs) were matched, but conditional logistic regressions failed to identify predictors for the emergence of Q151M.

Table 1. Characteristics of patients who achieved viral suppression (two consecutive viral load <50 copies/mL) and characteristics of the first successful treatment following detection of 69 insertion or Q151M.

Mutation, patient	Year of detection	Log_{10}HIV-1 RNA at detection, copies/mL	CD4+ cell count at detection, cells/µL	CDC stage	NRTI mutations at detection	Major[a] NNRTI mutations at detection	Major[a] PI mutations at detection	Year of first successful treatment	Successful treatment	Year of loss of follow-up
69 insertion										
#1	1995	4.4	50	C	M41L, 69 insertion, L210W, T215Y			2003	TDF EFV ddI	2009
#2	1995	4.2	118	B	M41L, 69 insertion, K70R, M184V, L210W, T215Y	Y188C		1999	NFV ZDV 3TC	2009
#3	1997	5.9	32	C	M41L, 69 insertion, L210W, T215Y		M46L, V82A, L90M			2001[b]
#4	1997	3.3	472	B	M41L, 69 insertion, M184I, T215Y			1997	SQV RTV NVP D4T	2009
#5	1997	4.3	315	C	M41L, 69 insertion, T215Y		I84V	2000	LPV IDV D4T 3TC	2009
#6	1999	3.1	180	B	M41L, 69 insertion, L210W, T215Y		M46I, T74P, I84V, L90M	2001	LPV EFV ddI 3TC	2009
#7	1999	5.1	139	C	M41L, 69 insertion, M184I, T215Y		M46L, V82A, I84V	2002	EFV ddI ABC	2009
#8	2001	4.2	415	B	D67N, 69 insertion, K70R, K219Q			2004	RTV EFV ATV 3TC	2009
#9	2002	4.8	542	B	M41L, 69 insertion, T215Y	Y181C	M46I, G48V, I84V, N88S, L90M			2009
#10	2003	5.7	13	B	M41L, 69 insertion, L74V, L210W, T215Y	L100I, K103N	M46I, L76V, V82F, L90M			2003[b]
#11	2004	4.8	198	B	M41L, 69 insertion, M184V, L210W, T215Y		M46I, I84V, L90M	2005	LPV EFV ZDV 3TC	2009
#12	2004	5.1	367	C	M41L, 69 insertion, L210W, T215Y		M46L, V82A, L90M			2009
#13	2007	2.9	358	B	M41L, 69 insertion, M184V, L210W, T215Y	K103N	I54L, I84V, L90M	2008	TDF RAL ETV ABC 3TC	2009
Q151M										
#1	1995	5.4	160	A	V75I, F77L, Y115F, F116Y, Q151M		Q58E			2001[b]
#2	1996	5.7	50	C	F77L, F116Y, Q151M		G48V, L90M			1997[b]
#3	1996	4.2	100	C	D67N, K70R, F77L, Q151M, T215F, K219E	V108I				2000[b]
#4	1996	3.5	162	B	F116Y, Q151M,			1999	NFV D4T 3TC	2009
#5	1996	4.4	133	B	K65R, V75I, F77L, Y115F, F116Y, Q151M, K219Q			2008	TDF RTV RAL MVC ETV DRV ZDV 3TC	2009

Table 1. (Continued)

Mutation, patient	Year of detection	Log_{10}HIV-1 RNA at detection, copies/mL	CD4+ cell count at detection, cells/µL	CDC stage	NRTI mutations at detection	Major[a] NNRTI mutations at detection	Major[a] PI mutations at detection	Year of first successful treatment	Successful treatment	Year of loss of follow-up
#6	1997	5.2	75	C	M41L, A62V, V75I, F77L, F116Y, Q151M, M184V					1998[b]
#7	1997	2.6	464	A	D67N, Q151M, K219Q					2001[b]
#8	1997	3.6	61	C	Q151M, M184V					2001[b]
#9	1997	3.8	476	C	D67N, F116Y, Q151M		Q58E	1997	IDV D4T 3TC	2009
#10	1998	5.1	15	C	A62V, V75I, F77L, F116Y, Q151M, M184V	K103N	M46L, G48V, V82A			2003[b]
#11	1998	5.0	8	C	M41L, D67N, F77L, F116Y, Q151M, M184V, L210W, T215Y		M46I, V82A			2000[b]
#12	1998	3.9	412	B	A62V, K65R, V75I, F77L, Y115F, F116Y, Q151M, M184V		D30N	1999	SQV RTV EFV D4T ABC	2007[b]
#13	1999	5.8	5	C	D67N, Q151M		V82A, I84V, L90M			2000[b]
#14	1999	5.3	23	C	D67N, K70R, F116Y, Q151M, M184V, K219E		V82A, L90M	2007	RTV RAL ETV DRV 3TC	2009
#15	2000	5.3	36	C	M41L, D67N, K70R, Q151M, M184V, T215F, K219E	K103N, V108I	I54L, V82A, L90M			2002[b]
#16	2000	4.9	481	B	A62V, V75I, F77L, F116Y, Q151M			2002	LPV EFV ZDV ABC 3TC	2009
#17	2001	2.5	259	C	A62V, F116Y, Q151M, M184V,		M46I	2002	EFV D4T 3TC	2003[b]
#18	2001	4.7	229	A	A62V, V75I, F77L, F116Y, Q151M		I84V, L90M	2003	LPV EFV APV 3TC	2009
#19	2001	5.0	253	C	A62V, K65R, D67N, V75I, F77L, Y115F, F116Y, Q151M, M184V, K219E		D30N, M46I, I54M	2002	SQV NVP LPV	2009
#20	2002	4.8	77	B	K65R, K70R, V75I, F77L, Y115F, F116Y, Q151M, K219E	K101E, Y181C, G190A	M46I, V82A	2004	TDF RTV ETV ABC	2009
#21	2003	3.9	191	C	D67N, K70R, F116Y, Q151M, M184V, K219Q	K103N	M46I, Q58E, V82F, L90M	2004		2006[b]
#22	2003	3.0	5	B	A62V, D67N, K70R, V75I, F77L, F116Y, Q151M, M184V, K219E	K103N, V108I	I50V, V82A, L90M	2006	TPV TDF T20 LPV DDI ZDV APV 3TC	2009
#23	2004	4.5	84	B	D67N, K70R, Y115F, F116Y, Q151M, M184V, K219Q	Y181C, Y188L	M46I, V82A	2007	TPV TDF RTV RAL ZDV 3TC	2009
#24	2004	3.9	461	B	F116Y, Q151M,		M46I, L90M	2006	RTV DRV	2008[b]
#25	2006	4.8	773	A	F116Y, Q151M	K103N, Y188L			TDF RTV FTC ATV	2009

Note. Viral suppression was defined as 2 consecutive viral loads <50 copies/mL. NRTI nucleoside reverse transcriptase inhibitor, NNRTI non-nucleoside reverse transcriptase inhibitor, PI protease inhibitor, 3TC, lamivudine; ABC, abacavir; APV, amprenavir; CDC, Centers for Disease Control and Prevention; D4T, stavudine; ddI, didanosine; DRV, darunavir; EFV, efavirenz; ETV, etravirine; HIV-1, human immunodeficiency virus type 1; IDV, indinavir; LPV, lopinavir; MVC, maraviroc; NFV, nelfinavir; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NVP, nevirapine; PI, protease inhibitor; RAL, raltegravir; RTV, ritanovir; SQV, saquinavir; T20, enfuvirtide; TDF, tenofovir; TPV, tipranavir; ZDV, zidovudine.

[a]Mutations printed in bold on the International AIDS Society-USA list.[12]

[b]Year of death

Virological response to salvage treatment upon emergence of MNR

Virological outcomes of patients with >1 follow-up HIV-1 RNA measurement following MNR detection were analyzed. The probability of ever achieving viral suppression was comparable between the 69 insertion group (9 [69.2%] of 13; 95% CI, 38.6%-90.9%) and the respective control subjects (15 [75.0%] of 20; 95% CI, 50.9%-91.3%). A large proportion of patients who received previously unseen drug classes achieved viral suppression (87.5% compared to 40.0% of other patients; *P*=0.217). As shown in table 1, all patients detected without major NNRTI or PI mutations achieved viral suppression (patients 1, 4, and 8). Five of 7 patients with either NNRTI or PI mutations achieved viral suppression with the remaining active non-NRTI drug class (patients 2, 5, 6, 7, and 11).

Viral suppression rates between the Q151M group (14 [56.0%] of 25; 95% CI, 34.9%-75.6%) and respective control subjects (27 [73.0%] of 37; 95% CI, 55.9%-86.2%) were similar. Generally, patients who achieved viral suppression had a higher median CD4+ cell count at detection (241 cells/μL vs 61 cells/μL; *P*=0.030), a lower viral load (median $\log_{10}$ RNA level, 4.5 copies/mL vs 5.1 copies/mL; *P*=0.089), a higher percentage of a previously unseen drug classes (88.9% vs 37.5%; *P*=0.033) and were detected later (median year, 1997 vs 2001; *P*=0.027). As illustrated in Table 1, most patients with Q151M detected before the introduction of HAART never achieved viral suppression (patients 1-3 and 6-8). Four of 11 patients detected in the HAART era prior to the approval of enfuvirtide (T20) (1998-2002) never had a successful treatment (patients 10, 11, 13, and 15). All of these patients had low CD4+ cell counts (<50 cells/μL) and extensive PI and/or NNRTI mutations. Most of the other patients had PI mutations but achieved viral suppression with a regimen containing NNRTIs (patients 12, 14, and 16-20).

Survival after detection of MNR

The crude incidence of mortality after detection of the 69 insertion was 1.9 deaths (95% CI, 0.2-6.9) per 100 person-years of follow-up, compared with 6.3 deaths (95% CI, 2.9-12.0) per 100 person-years of follow-up among control subjects (≥3 TAMs) [figure 1]. Of the 2 deaths noted in the 69 insertion group, 1 death was HIV-related and the other cause of the other death was unknown. The risk of mortality was not significantly different between patients with the 69 insertion and those with ≥3 TAMs

(univariable hazard ratio [HR], 0.3 [95% CI, 0.1-1.6]; *P*=0.178). The small number of events did not allow stratified or multivariable models.

Patients with Q151M detected tended to have a higher crude incidence of 9.8 deaths per 100 person-years (95% CI, 5.3-16.4) compared with 5.8 deaths per 100 person-years (95% CI, 3.3-9.4) in control subjects (≥3 TAMs) [Figure 1]. HIV infection was the cause of death for 6 (42.9%) of14 patients from the Q151M group and 7 (43.8%) of 16 control subjects. Additional causes of death reported in the Q151M group were neoplasm (14.3%), cardio-vascular diseases (14.3%), chronic hepatitis C (7.1%), suicide (7.1%) or unknown (14.3%). The detection of Q151M was associated with increased mortality but was of marginal statistical significance in the univariable (HR, 2.7 [95% CI, 0.9-8.0]; *P*=0.075) and multivariable model, adjusted for sex, ethnicity, risk group, CD4+ cell count, and age (HR, 7.5 [95% CI, 0.9-64.6]; *P*=0.068).

Sensitivity analyses including only those deaths associated with HIV infection (univariable HR, 2.6 [95% CI, 0.6-10.4]; *P*=0.189) or by additionally matching case patients and control subjects by CD4+ cell count at time of detection of Q151M or $\geq$3 TAMs showed similar results (univariable HR, 3.2 [95% CI: 1.0-10.9]; *P*=0.058; multivariable HR: 6.2 [95% CI, 0.7-56.0]; *P*=0.105).

Discussion

Because the prevalence of MNR is increasing in resource-limited countries, and because 69 insertion and Q151M affect the activity of an entire drug class, it is of great importance to identify risk factors and to optimize treatment strategies.

Our study currently represents the largest longitudinal dataset with full treatment history available. We found high evidence of forward transmission of 69 insertions. This finding is of relevance, because the presence of 69 insertion results in a substantial reduction of treatment options, which can be devastating in settings with limited access to potent salvage therapies.

Moreover, our study identified a significant association of ddI exposure with the emergence of the 69 insertion.[13] No specific NRTI was associated with the emergence of Q151M. Our study did not confirm a previously reported negative association of lamivudine with Q151M,[2, 14] nor was d4T exposure correlated with an increased risk for Q151M (data not shown).

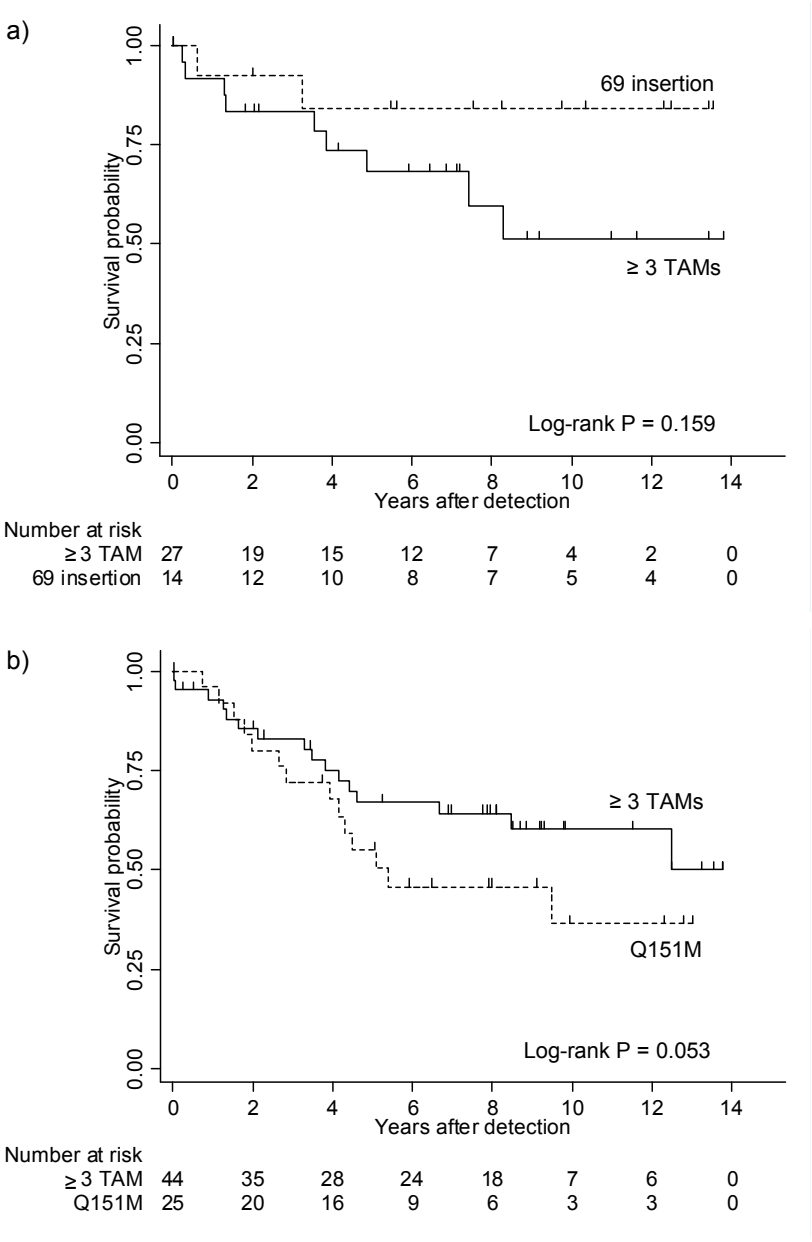

Figure 1. Kaplan-Meier curves showing survival after detection of 69 insertion (a) or Q151M (b). Patients detected with ≥3 thymidine analogue mutations (TAMs) were matched (2:1) for comparison. Log-rank test was stratified for matched pairs.

Of note, MNR mutations were never detected in patients who were exclusively treated with HAART. This is in contrast to the high prevalence of MNR mutations observed in resource-limited settings.[7, 8] Free access to potent antiretroviral drugs in Switzerland and close monitoring are the most likely explanations for this difference.

Moreover, this study widened the very limited knowledge for treatment strategies of patients detected with MNR. These patients can be successfully treated if potent drugs, such as boosted PI, raltegravir or T20 are available. The descriptive analysis

of therapy success further suggests that extensive resistance to NNRTIs and PIs, as well as a low CD4+ cell count at the time of detection of MNR mutations, were prognostic unfavourable.[15]

Patients with viruses possessing Q151M tended to have an increased mortality risk compared with patients with ≥3 TAMs. Although this finding was robust throughout several sensitivity analyses, conclusions regarding causality between Q151M and death should be drawn with care.

This study has some limitations. Even though our sample is the largest study so far addressing MNR, it is still limited in power.[11] Our study was restricted to subtype B-infected individuals, and results may therefore not be readily transferable to other subtypes.

Taken together, our data indicate that modern antiretroviral therapies in combination with adequate viral monitoring are able to prevent the emergence of MNR mutations in developed settings. In Switzerland, detected MNR mutations are mainly a relic of the mono- or dual-NRTI therapy era, although 2 cases of transmitted MNR were observed. This analysis further demonstrates that salvage treatment can be successful even when MNR mutations are present if at least 1 previously unseen drug class is available. Today, the development of MNR seems to be becoming an emerging problem in resource-limited settings, where most patients are infected with non-subtype B strains. Thus, additional studies are needed to investigate whether our findings are also true for non-subtype B infections.

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Chapter 5

Mutational pattern of multi-nucleoside resistance mutations

Manuscript submitted

Polymorphic mutations associated with the emergence of the multi-nucleoside/tide resistance mutations 69 insertion and Q151M

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AUS contributed to the study design, performed the statistical analysis and drafted the article.

Abstract

Background

We hypothesized that polymorphic mutations exist that are associated with the emergence of the multi-nucleoside resistance mutations (MNR), 69 insertion and Q151M.

Methods

The Swiss HIV Cohort Study (SHCS) was screened and the frequencies of polymorphic mutations in HIV-1 (subtype B) were compared between patients detected with the 69 insertion (n=17), Q151M (n=29), ≥2 thymidine analogue mutations (TAM) 1 (n=400) or ≥2 TAM 2 (n=249). Logistic regressions adjusted for the antiretroviral treatment history were performed to analyze the association of the polymorphic mutations with MNR.

Results

The 69 insertion and TAM 1 were strongly associated and occurred in 94.1% (16/17) together. The 69 insertion seemed to emerge as a consequence of the TAM 1 pathway (median years until detection: 6.8 compared to 4.4 for ≥2 TAM 1, *P* Wilcoxon=0.009). Frequencies of 8 polymorphic mutations (K43E, V60I, S68G, S162C, T165I, I202V, R211K, F214L) were significantly different between groups. Logistic regression showed that F214L and V60I were associated with the emergence of Q151M/TAM 2 opposed to 69 insertion/TAM 1. S68G, T165I and I202V were associated with Q151M instead of TAM 2.

Conclusion

Besides antiretroviral therapy polymorphic mutations may contribute to the emergence of specific MNR mutations.

Introduction

The emergence of resistance mutations during antiretroviral therapy (ART) of human immunodeficiency virus type 1 (HIV-1) infections is an important cause for treatment failure. The 69 insertion and the Q151M mutation are two rare drug-associated genotypic modifications causing multi-nucleoside/tide resistance (MNR).[1-5] The prevalence among several European studies was <3.5%, but recent studies from resource-limited countries reported a higher prevalence.[6-9] Earlier studies showed that the 69 insertion often co-occurred with mutations from the thymidine analogue mutation (TAM) 1 pathway (M41L, L210W and T215W).[10, 11] Q151M has a completely different resistance pattern and is usually accompanied by two or more accessory mutations (A62V, V75I, F77L, and F116Y) that compensate the negative impact of Q151M on viral replication.[1] A possible association of the Q151M and the TAM pathways was discussed controversially.[10, 12, 13] In developed countries, MNR mutations mostly have occurred during outdated single class nucleoside reverse transcriptase inhibitor (NRTI) therapy. In addition, the 69 insertion has been associated with didanosine exposure.[5, 14, 15] Otherwise very little is known about risk factors for the emergence of MNR, mostly due to the rare occurrence of MNR mutations and hence the limited sample size for analysis.[13, 14]

Previous studies have already demonstrated the existence of polymorphic mutations that are strongly associated with the emergence of TAM 1 as opposed to TAM 2 (e.g. F214L).[16-18] Therefore, we hypothesized that, besides exposure to specific drugs, the emergence of the different MNR profiles, 69 insertion and Q151M, may also depend on particular genomic signatures of the virus in the polymerase region. We aimed to identify such mutations using data from the Swiss HIV Cohort study (SHCS) and the SHCS drug resistance data base.

Methods

General procedure

To identify mutations associated with the emergence of MNR, we proceeded as follows: First, we determined whether the emergence of the two MNR profiles, 69 insertion and Q151M, are linked to specific TAM patterns. In particular, we were interested in finding out whether the MNR mutations could occur independently from TAMs or whether they mark an endpoint of a specific TAM pathway (analysis step 1). The result from this analysis then allowed us to form appropriate groupings for

MNR/TAM pathways to be used in subsequent analyses (henceforth named MNR/TAM groups). Next we identified polymorphic mutations, which were not significantly more prevalent in patients with treatment exposure compared to individuals without treatment exposure. This initial set of polymorphic mutations was further restricted to mutations, which were present at significantly different proportions across the MNR/TAM groups defined in the first analysis, thus suggesting an accumulation of polymorphic mutations in certain MNR/TAM groups (analysis step 2). In a final analysis, the predictive values of the found polymorphic mutations were studied (analysis step 3).

Statistical analyses were performed with Stata 11 SE (StataCorp, College Station, TX). All confidence intervals (CI) are 95% CI and the level of significance was set at 0.05 unless indicated otherwise.

Data and study population

Data from the SHCS resistance data base were analyzed, which contains all genotypic HIV resistance tests performed by the four authorized laboratories in Switzerland, stored in SmartGene's (Zug, Switzerland) Integrated Database Network System (IDNS version 3.5.7).[19] Additionally, clinical data came from the SHCS, which is a nationwide, clinic-based cohort study with continuous enrolment and semi-annual study visits.[20, 21] The SHCS has been approved by ethical committees of all participating institutions and written informed consent has been obtained from participants. Only sequences from therapy exposed individuals infected with subtype B viruses were considered for analysis, because of very low numbers of 69 insertion (n=1) and Q151M (n=5) among non-B strains.

Association of the 69 insertion and Q151M with TAMs (analysis step 1)

To start with, we built four groups representing the particular resistance pathways: 1) patients with the 69 insertion detected, 2) patients with the Q151M detected, 3) patients with ≥2 TAM 1 (M41L, L210W, T215Y), and 4) patients with ≥2 TAM 2 (D67N, K70R, T215F, K219K/E) detected. Sequences with both, TAM 1 and TAM 2, were excluded (n=495). Sequences with 69 insertion and TAMs were allocated to the 69 insertion group and sequences with Q151M and TAMs to the Q151M group.

In order to assess whether MNR mutations can emerge independently of TAMs, the percentage of co-occurrence of the 69 insertion or Q151M with TAMs of group 1 or 2

was compared. In addition, we investigated the possible chronological order of the TAM and MNR mutations. For this purpose, the time from therapy initiation until detection of either TAMs or MNR mutations was compared between the different MNR profiles and the two TAM pathways. In particular, we aimed to study whether MNR mutations tended to follow TAMs, which would support the hypothesis that the MNR mutations emerge as the end point of the respective TAM pathway. Empirical distributions of time until mutation detection (described by median and interquartile ranges) were compared by use of the Wilcoxon rank sum test. Based on these analyses we regrouped the four initial MNR or TAM groups to represent possible pathways.

Identification of polymorphic mutations associated with different MNR/TAM groups (analysis step 2)

To identify polymorphic mutations, we compared the frequency of all reverse transcriptase (RT) mutations between treatment-exposed patients (detected with 69 insertion, Q151M, ≥2 TAM 1 or ≥2 TAM 2) and an equal number of randomly selected sequences from treatment-naïve patients. For the definition of a polymorphism, only amino acid changes with a prevalence of at least 3% among treatment-naïve patients were considered. In addition, the prevalence of the respective mutation among treatment-experienced patients was not allowed to be significantly higher compared to the frequency among treatment-naïve patients. Analogous to algorithms used in analyses of non-inferiority clinical trials [22], an upper limit for an allowed difference in prevalence of specific mutations between treatment-naive and treatment-exposed individuals was determined to still be considered equivalent (or non-superior), which was set at 5% in this study. If the 95% CI of the difference in prevalence did not include this margin (i.e. was superior) then this mutation was discarded from the list of potential polymorphisms, otherwise the mutation was included. To verify that the identification of polymorphisms were not influenced unduly by our dataset, we applied the same selection criteria (i.e. >3% frequency among treatment-naive and <5% difference) to genotypic data from the Stanford database by querying the Genotype-Treatment Correlations tool (http://hivdb.stanford.edu/cgi-bin/RTMutSummary.cgi).

Of this initial set of polymorphic mutations those were selected that varied significantly across the MNR/TAM groups defined in step 1. Significance was

assessed by use of Fisher's exact tests and Benjamini-Hochberg correction with a false-discovery rate of 5% to adjust for multiple testing.

The associations of polymorphic mutations with specific MNR/TAM groups were checked further by use of multivariable logistic regression models adjusted for the exposure to specific NRTIs (ever exposed). Variables (polymorphic mutations and treatment exposure) were included in the multivariable model if the *P* value in the univariable model was <0.1.

Predictive values of polymorphic mutations (analysis step 3)

The predictive values of the identified polymorphic mutations were assessed by performing non-parametric receiver operating characteristic (ROC) analyses defining the sensitivity, specificity, number of correctly classified and the area under ROC curve (AUC).

To further validate the predictive value of polymorphisms identified in analysis step 2, we queried the Stanford database Genotype-Treatment Correlations tool for sequence pairs consisting of one genotype performed before treatment exposure and one obtained after any exposure to zidovudine and/ or stavudine (see results). Sequence pairs were included in the analysis if the sequence taken after treatment exposure either carried the 69 insertion, ≥2 TAM 1, ≥2 TAM 2, or Q151M, analogous to our initial selection criteria for sequences from the SHCS drug resistance database. The same analytic methods for prediction performance assessment were used as for the SHCS dataset.

Results

Association of TAM 1, TAM 2, 69 insertion and Q151M (analysis step 1)

The SHCS was screened for genotypic resistance tests (GRT) from treatment-experienced patients. A total of 3335 subtype B sequences were selected. The 69 insertion and the Q151M mutation occurred very rarely, the prevalence was 0.5% (n=17/3335) and 0.9% (n=29/3335), respectively.

Previous studies suggested an association of the 69 insertion with the TAM 1 pathway. This finding was confirmed in our study. As shown in table 1, the 69 insertion co-occurred in 16/17 cases (94.1%) with ≥1 TAM 1 and rarely with ≥1 TAM 2 (2/17, 11.8%). In contrast to the 69 insertion, the Q151M mutation co-occurred very rarely with ≥1 TAM 1 (5/29, 17.2%), whereas TAM 2 were found frequently (16/29,

55.2%). These impressions were reinforced when checking the actual nucleotide sequences for ambiguous base calls at positions associated with TAM, 69 insertion and Q151M.[23] The presence of both wild type and mutant virus is suggestive for the presence of separate viral strains, which has been described previously for incompatible mutations such as TAM 1 and K65R.[24] Indeed, 2 out of 5 patients with Q151M and TAM 1 also harboured virus with wild type at these amino acid positions. The infrequent co-occurrence of Q151M and TAM 1 on the same strain (3 of 29, 10%) indicates strong fitness interactions between these mutations. For the sequences containing the 69 insertion no ambiguous base calls were detected at relevant positions. Of further note, while the 69 insertion only once occurred in the absence of TAM 1, there were 12 (41%) cases of Q151M without the presence of any TAMs (and TAM 2 in particular). This finding indicates that Q151M emergence may be independent of the TAM 2 pathway.

Table 1. Occurrence of the 69insertion, Q151M and thymidine-analogue mutations (TAM) 1 and TAM 2.

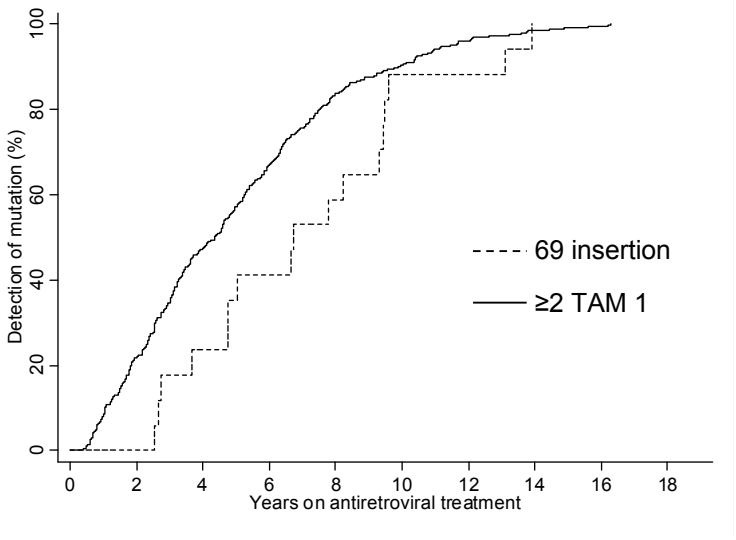
Mutation	69 insertion(n=17)	Q151M (n=29)
TAM 1 (≥1 mutation)	16 (94.1%)	5 (17.2%)
M41L	16 (94.1%)	4 (13.8%)
L210W	10 (58.8%)	2 (6.9%)
T215Y	16 (94.1%)	2 (6.9%)
TAM 2 (≥1 mutation)	2 (11.8%)	16 (55.2%)
D67N	1 (5.9%)	14 (48.3%)
K70R	2 (11.8%)	6 (20.7%)
T215F	0 (0.0%)	3 (10.3%)
K219E	0 (0.0%)	6 (20.7%)
K219Q	1 (5.9%)	5 (17.2%)
No TAMs	0 (0%)	12 (41.4%)

Next, to better understand the chronological order of the emergence of resistance mutations, the time until detection of MNR and ≥2 TAMs was analyzed. Given the results from above we hypothesized that the 69 insertions may result from the TAM 1 pathway after prolonged exposure to ART. The empirical distributions of time to occurrence of the 69 insertion or ≥2 TAM 1 (figure 1a) showed that the overall time on ART until detection of 69 insertion (median [IQR]: 6.8 years [4.7-9.4]) was longer compared to ≥2 TAM 1 (median [IQR]: 4.4 years [2.3-6.9], *P* Wilcoxon=0.009). These findings support the notion that the 69 insertion results out of the TAM 1 pathway. The same type of analysis however showed no time dependent difference between the occurrence of Q151M and TAM 2 mutations: 5.5 years (IQR: 3.9-6.4) and 5.1 years (IQR: 2.9-7.0, *P*=0.566), respectively (figure 1b). Additionally, no difference

was found between patients detected exclusively with mutations from the Q151M pattern (median years [IQR]: 5.0 [1.9-6.1]) and patients detected with a combination of the Q151M and TAM 2 mutations (median years [IQR]: 5.9 [4.5-8.8, *P*=0.136]).

On the basis of these results we established the following hypotheses for the dependencies between TAM and MNR. The 69 insertion emerges out of the TAM 1 pathway and is strongly discriminated against by TAM 2 mutations. In contrast, Q151M is selected against by TAM 1 mutations, but there is no strict association with TAM 2 mutations. Thus, in the following analyses we considered TAM 2 and Q151M as separate MNR/TAM categories, and a third group consisting of TAM 1 and 69 insertion.

a)



b)

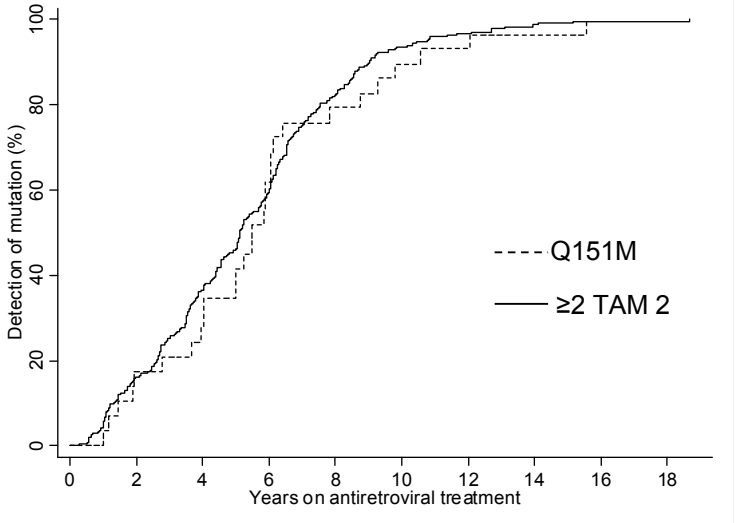


Figure 1. Time on antiretroviral treatment until the detection of 69 insertion (n=17) and ≥2 thymidine analogue mutations 1 (TAM 1, n=400) [a], or Q151M (n=29) and ≥2 TAMs 2 (n=249) [b]

Polymorphic mutations associated with the emergence of MNR mutations (analysis step 2)

To identify polymorphic mutations associated with the emergence of MNR mutations, we screened all RT mutations and compared their frequencies between treatment-naive individuals and all treatment-experienced individuals from the three MNR/TAM groups combined.

We identified 95 mutations which fulfilled the criteria for a polymorphic mutation, meaning that the frequency among treatment-naïve patients was >3% and the 95% CI of the difference between treatment-exposed and treatment-naïve patients did not contain the 5% margin. Out of these 95 mutations, 8 (8.4%) showed statistically significant differences in proportions across the three MNR/TAM groups after adjustment for multiple testing: K43E, V60I, S68G, S162C, T165I, I202V, R211K and F214L (table 2). We checked these findings with data from the Stanford database, which included 12,172 sequences from treatment-naïve and 9,101 sequences from treatment-experienced patients. With the exception of K43E all mutations classified as polymorphisms by our algorithm were confirmed, meaning that they showed a prevalence of >3% among treatment-naïve patients and the differences in prevalence relative to treatment-exposed sequences was <5%.

Table 2. Polymorphic mutations associated with the 69 insertion/ thymidine-analogue mutations (TAM) 1, Q151M or TAM 2.

Mutation	69 insertion/ ≥2TAM 1 (n=417)	Q151M (n=29)	≥2 TAM 2 (n=249)	*P* exact	Treatment experienced (n=696)	Treatment naïve (n=696)	Difference (95% confidence interval)
K43E	38 (9.1%)	0 (0.0%)	4 (1.6%)	<0.001	42 (6.0%)	27 (3.9%)	2.2% (-0.1 to 4.4)
V60I	61 (14.6%)	7 (24.1%)	63 (25.3%)	0.002	131 (18.9%)	145 (20.9%)	-2.0% (-6.2 to 2.2)
S68G	28 (6.7%)	8 (27.6%)	4 (1.6%)	<0.001	40 (5.8%)	32 (4.6%)	1.2% (-1.2 to 3.5)
S162C	51 (12.2%)	3 (10.3%)	11 (4.4%)	0.002	65 (9.3%)	119 (17.1%)	-7.8% (-1.1 to -4.2)
T165I	5 (1.2%)	5 (17.2%)	5 (2.0%)	<0.001	15 (2.2%)	38 (5.5%)	-3.3% (-.5.3 to -1.3)
I202V	30 (7.2%)	12 (41.4%)	36 (14.5%)	<0.001	78 (11.2%)	78 (11.2%)	0.0% (-3.3to 3.3)
R211K	230 (55.2%)	13 (44.8%)	91 (36.5%)	<0.001	334 (48.1%)	361 (51.9%)	-3.9% (-9.1 to 1.4)
F214L	9 (2.2%)	8 (27.6%)	77 (30.9%)	<0.001	94 (13.5%)	115 (16.6%)	-3.0% (-6.8 to 0.7)

The polymorphism F214L showed the most pronounced difference between the MNR/TAM groups. It occurred very rarely (2.2%) in the 69 insertion/TAM 1 group, but frequently in the Q151M (27.6%) or TAM 2 group (30.9%), which provides evidence that F214L might direct viral evolution towards the Q151M- and TAM 2-pathways as opposed to the TAM 1 pathway. Compared with the 69 insertion/TAM 1- and the TAM 2-group, S68G and I202V co-occurred frequently with Q151M, in 27.6% and 41.4% of cases, but only at 6.7% and 7.2% in the 69 insertion/TAM 1 group and at 1.6% and

14.5% in the TAM 2 group, respectively. From these comparisons and the results shown in table 1 we inferred that there may exist a similarity between the Q151M and the TAM 2 group, but a pronounced dissimilarity between these two groups with the 69 insertion/TAM 1 group in terms of polymorphism profiles. We hypothesized that there may exist an early split in pathways between TAM 1 and the other two MNR/TAM groups, and a later split between TAM 2 and Q151M, as outlined graphically in figure 2. Accordingly, modelling was performed in two sequential steps. The first step consisted of a multivariable comparison of the 69 insertion group with a pooled group of TAM2 and Q151M with respect to polymorphisms and therapy exposures. Subsequently, factors separating the TAM 2 and Q151M groups were identified by repeating the multivariable modelling analysis on these two groups only.

Table 3. Univariable and multivariable logistic regression comparing patients detected with 69 insertion/≥2 thymidine-analogue mutations (TAM) 1 (reference) with Q151M/ ≥2 TAM 2.

	69 insertion / ≥2 TAM 1 (n=417)	Q151M / ≥2 TAM 2 (n=278)	Univariable		Multivariable	
Characteristics			OR (95% CI)	*P*	OR (95% CI)	*P*
K43E	38 (9.1%)	4 (1.4%)	0.1 (0.1-0.4)	<0.001	0.1 (0.0-0.4)	0.001
V60I	61 (14.6%)	70 (25.2%)	2.0 (1.3-2.9)	0.001	1.9 (1.2-2.9)	0.006
S68G	28 (6.7%)	12 (4.3%)	0.6 (0.3-1.3)	0.187	-	
S162C	51 (12.2%)	14 (5.0%)	0.4 (0.2-0.7)	0.002	0.4 (0.2-0.8)	0.016
T165I	5 (1.2%)	10 (3.6%)	3.1 (1.0-9.1)	0.042	2.3 (0.6-8.4)	0.217
I202V	30 (7.2%)	48 (17.3%)	2.7 (1.7-4.4)	<0.001	2.5 (1.5-4.4)	0.001
R211K	230 (55.2%)	104 (37.4%)	0.5 (0.4-0.7)	<0.001	0.5 (0.4-0.8)	0.001
F214L	9 (2.2%)	85 (30.6%)	20.0 (9.8-40.5)	<0.001	19.0 (9.0-40.1)	<0.001
Ever used 3TC	297 (71.2%)	211 (75.9%)	1.3 (0.9-1.8)	0.174	-	
Ever used ABC	79 (18.9%)	69 (24.8%)	1.4 (1.0-2.0)	0.064	1.2 (0.8-1.9)	0.373
Ever used ZDV	387 (92.8%)	254 (91.4%)	0.8 (0.5-1.4)	0.488		
Ever used D4T	239 (57.3%)	178 (64.0%)	1.3 (1.0-1.8)	0.077	1.3 (0.9-2.0)	0.137
Ever used ddC	117 (28.1%)	58 (20.9%)	0.7 (0.5-1.0)	0.033	0.6 (0.4-1.0)	0.034
Ever used ddI	262 (62.8%)	157 (56.5%)	0.8 (0.6-1.0)	0.094	0.7 (0.5-1.0)	0.061
Ever used TDF	29 (7.0%)	17 (6.1%)	0.9 (0.5-1.6)	0.663	-	

OR, odds ratio; CI confidence interval; 3TC, lamivudine; ABC, abacavir; ZDV, zidovudine; D4T, stavudine; ddC, zalcitabine; ddI, didanosine; TDF, tenofovir

The first logistic regression comparing the pooled 69 insertion/TAM 1 groups with the pooled Q151M/TAM 2 groups (table 3) confirmed the strong association of the polymorphic mutation F214L with the emergence of Q151M and or ≥2 TAM 2 (univariable odds ratio (OR): 20.0, 95% CI: 9.8-40.5, *P*<0.001; multivariable OR: 19.0, 95% CI: 9.0-40.1, *P*<0.001). Moreover, V60I and I202V were also associated with the emergence of Q151M/≥2 TAM 2, whereas K43E and R211K were negatively associated. In the second step aiming at finding polymorphisms which may influence

the emergence of Q151M as opposed to TAM 2 mutations, S68G was strongly associated with the occurrence of Q151M (univariable OR: 23.2, 95% CI: 6.5-83.9, *P*<0.001; multivariable OR: 18.1, 95% CI: 4.0-81.3, *P*<0.001). Additionally, S162C and I202V were positively associated with the occurrence of Q151M. Of note, this analysis also suggests a role of stavudine use and possibly also zalcitabine in the emergence of the MNR mutation Q151M (table 4).

Table 4. Univariable and multivariable logistic regression comparing patients detected with ≥2 thymidine-analogue mutations (TAM) 2 (reference) and Q151M.

	≥2 TAM 2 (n=249)	Q151M (n=29)	Univariable		Multivariable	
Characteristics			OR (95% CI)	*P*	OR (95% CI)	*P*
K43E	4 (1.6%)	0 (0%)	-		-	
V60I	63 (25.3%)	7 (24.1%)	0.9 (0.4-2.3)	0.891	-	
S68G	4 (1.6%)	8 (27.6%)	23.3 (6.5-83.9)	<0.001	18.1 (4.0-81.3)	<0.001
S162C	11 (4.4%)	3 (10.3%)	2.5 (0.7-9.5)	0.181	-	
T165I	5 (2.0%)	5 (1.2%)	10.2 (2.7- 37.6)	0.001	9.2 (2.0-42.4)	0.005
I202V	36 (14.5%)	12 (41.4%)	4.2 (1.8-9.5)	0.001	6.2 (2.2-17.6)	0.001
R211K	91 (36.6%)	13 (44.8%)	1.4 (0.6-3.1)	0.385		
F214L	77 (30.9%)	8 (27.6%)	0.9 (0.4-2.0)	0.712	-	
Ever used 3TC	188 (75.5%)	23 (79.3%)	1.2 (0.5-3.2)	0.650	-	
Ever used ABC	60 (24.1%)	9 (31.0%)	1.4 (0.6-3.3)	0.415	-	
Ever used ZDV	228 (91.6%)	26 (89.7%)	0.8 (0.2-2.9)	0.729	-	
Ever used D4T	152 (61.0%)	26 (89.7%)	5.5 (1.6-18.8)	0.006	6.3 (1.5-25.9)	0.011
Ever used ddC	46 (18.5%)	12 (41.4%)	3.1 (1.4-7.0)	0.006	4.3 (1.6-11.7)	0.005
Ever used ddI	134 (53.8%)	23 (79.3%)	3.3 (1.3-8.4)	0.012	1.5 (0.5-4.3)	0.500
Ever used TDF	14 (5.6%)	3 (10.3%)	1.9 (0.5-7.2)	0.323	-	

OR, odds ratio; CI confidence interval; 3TC, lamivudine; ABC, abacavir; ZDV, zidovudine; D4T, stavudine; ddC, zalcitabine; ddI, didanosine; TDF, tenofovir

Predictive values of polymorphic mutations (analysis step 3)

The polymorphism with the highest sensitivity (30.6%) and specificity (97.8%) to predict Q151M/TAM 2 as opposed to 69 insertion/TAM 1 was F214L, with an area under the curve (AUC) of 0.64. Having this mutation predicted the correct pathway (i.e. Q151M/TAM 2) in 71.0% of cases. The sensitivity, specificity and the percentage of correctly classified of V60I and I202V were 17.3% and 25.2%, 92.8% and 85.4%, and 62.6% and 61.3%, respectively.

In the second analysis comparing only the TAM 2 and Q151M groups, the sensitivity and specificity of S68G to predict the TAM 2 pathway was 27.6% and 98.4%, respectively. The percentage of correctly classified was 91.0% and the area under ROC curve 0.74. For T165I and I202V were sensitivity 17.2% and 41.4%, specificity 98.0% and 85.5%, and percentage of correctly classified 89.6% and 80.9%, respectively.

For the external validation 36 sequence pairs from the Stanford database could be included , of which 8 had the Q151M mutation, 21 had ≥2 TAM 1, 22 ≥2 TAM 2. The 69 insertion was not observed. Interestingly, the polymorphisms F214L and V60I also showed the best prediction performance in this new sample for Q151M/TAM 2 (supplementary table 1), with 64% and 67% of samples correctly classified, respectively. Due to the very small numbers of S68G (n=1), T165I (n=1), and I202V (n=0) mutations in treatment-naive samples no meaningful comparisons could be performed between the Q151M and the TAM 2 group.

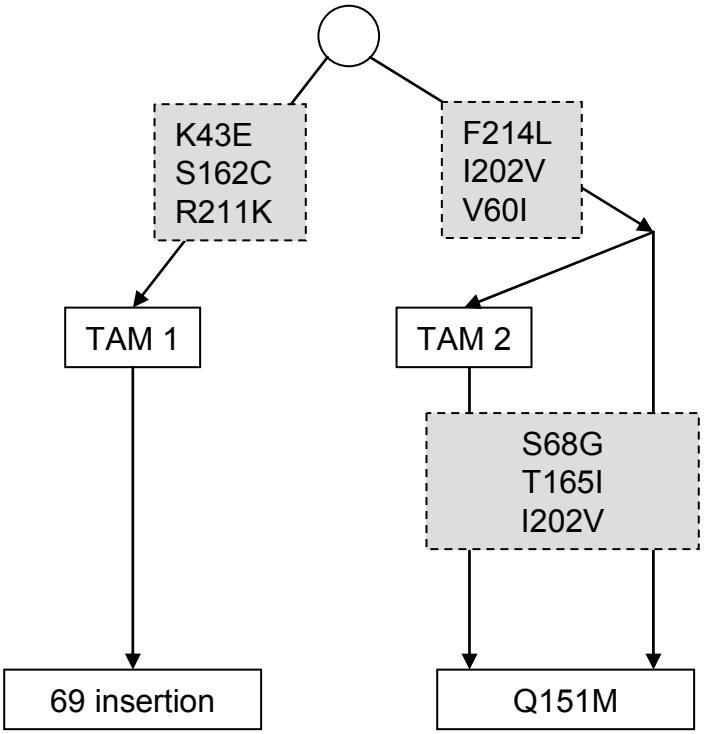


Figure 2. Polymorphic mutations associated with different resistance pathways.

Discussion

The present study aimed to better characterize possible viral genetic signatures associated with the emergence of MNR profiles. Specifically, it was investigated whether particular polymorphic mutations are associated with the occurrence of 69 insertion and Q151M. The study confirmed the cluster of the 69 insertion with the TAM 1 pathway. Among others, the polymorphism F214L was found to be strongly positively associated with the Q151M/TAM 2 pathway, but negatively with the 69 insertion/TAM 1 pathway. S68G, T165I and I202V were more common among

patients detected with Q151M in contrast to patients with TAM 2. In figure 2, the suggested resistance pathways are summarized.

F214L is a polymorphism known to direct resistance pathways.[16, 25] This is the first study showing an association with the emergence of Q151M. The high percentage of F214L in samples detected with the Q151M mutation is probably due to the negative association with the TAM 1 pathway. On a structural level, F214L is too far away from the Q151M pattern to interact directly. However, indirect effects via other amino acids can not formally be excluded. We showed a strong correlation between S68G and Q151M.[13] S68G was commonly observed together with the Q151M mutations. The prevalence of S68G among treatment-naïve patients was 4.5% compared to 5.8% among treatment-experienced patients, we can not definitively exclude an association with the treatment.[13, 26] S68G is known to partially compensate the negative impact on the viral replication of Q151L that is a potential intermediate of the Q151M mutation with a strongly decreased viral replication capacity.[27, 28] The polymorphic mutation I202V was found to be positively associated with the Q151M pathway. T165I and I202V might be an important factor directing viral evolution towards Q151M in contrast to TAM 2.

Our study is limited by the small sample size, but to date it is the largest study addressing the topic of MNR.[13, 14] Overall, the predictive values of polymorphisms for the emergence of specific pathways were quite low. This is not so surprising, given that the emergence or resistance mutations is also strongly influence by antiretroviral therapy, which was difficult to adjust for in this analysis. It should be noted however that we have been able to reproduce some of our main findings in an independent, strictly selected dataset, namely the negative associations of the polymorphisms F214L and V60I with the TAM 1/69 insertion pathway. Whether polymorphic mutations can favour the emergence of Q151M mutation is less clear from this analysis. While we found solid evidence for a distinct 69 insertion/TAM 1 pathway, there was no clear separation between Q151M and TAM 2 mutations. Although Q151M and TAM 2 can occur independently, these mutations do not seem mutually exclusive, and the emergence of Q151M may largely be driven be factors like treatment.

To conclude, this is the first study which found evidence for a dependency of MNR emergence on the genomic background of the HIV polymerase. The polymorphisms F214L and V60I were found to direct viral evolution towards Q151M- and TAM 2-

pathway in contrast to the 69 insertion/TAM 1-pathway. Other genotypic changes, such as S68G, T165I and I202V, were strongly associated with the emergence of Q151M, but their role was less clear. Nevertheless, a better understanding of the processes leading up to the emergence of MNR mutations is of great relevance in light of their negative clinical impact and the increasing MNR prevalence in resource limited settings.[29, 30] Similar studies in less developed settings and with subtypes other than subtype B are clearly warranted.

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Supplementary

Supplementary table 1. Validation of polymorphisms predicting for the thymidine analogue mutation (TAM) 1 pathway. Sequences were obtained from the Stanford database Genotype-Treatment Correlations tool consisting of one genotype performed before treatment exposure and one obtained after any exposure to zidovudine and/ or stavudine.

	TAM 1 (n=21)	no TAM 1 (n=15)	Sensitivity	Specificity	% Correctly classified	AUC
K43E	0	0	-	-	-	-
V60I	1 (6.7%)	4 (19%)	26.7%	95.2%	66.7%	0.61
S68G	1 (6.7%)	0	-	-	-	-
S162C	3 (20%)	2 (9.5%)	13.3%	85.7%	55.6%	0.50
T165I	1 (6.7%)	1 (4.8%)	7.0%	95.2%	58.3%	0.51
I202V	1 (6.7%)	0	-	-	-	-
R211K	7 (46.7%)	2 (9.5%)	13.0%	66.7%	44.4%	0.40
F214L	1 (6.7%)	3 (14.3%)	20.0%	95.2%	63.9%	0.58

AUC, area under ROC curve

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Chapter 6

Impact of HIV-1 subtype on cART response

In press in Clinical Infectious Diseases

Improved long-term virological outcome in Caucasians infected with HIV-1 non-B subtypes compared to subtype B

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AUS designed the study, performed the statistical analysis and drafted the article.

Abstract

Background

Antiretroviral compounds were predominantly studied in HIV-1 subtype B, but only approximately 10% of infections worldwide are caused by this subtype. Analyzing the impact of different HIV subtypes on treatment outcome is important.

Methods

The effect of subtype B and non-B on the time to virological failure while taking combination antiretroviral therapy (cART) was analyzed. Previous studies addressing this question were limited by the strong correlation of subtype and ethnicity. Our analysis was restricted to Caucasians from the Swiss HIV Cohort Study who started cART between 1996 and 2009. Cox regression models were performed, adjusted for age, sex, transmission category, first cART, baseline CD4 cells and HIV RNA, and stratified for previous mono/dual nucleoside reverse transcriptase inhibitor treatment.

Results

4729 patients infected with subtype B and 539 with non-B were included. Most prevalent non-B subtypes were CRF02_AG (23.8%), A (23.4%), C (12.8%) and CRF01_AE (12.6%). The incidence of a virological failure was higher in patients with subtype B (4.3 [95% CI: 4.0-4.5] failures/100 person-years) compared with non-B (1.8 [1.4-2.4]). Cox regression models confirmed that patients infected with non-B subtypes had a lower risk for virological failure compared with subtype B (univariable HR: 0.39 [0.30-0.52], $P<0.001$; multivariable HR: 0.68, [0.51-0.91], $P=0.009$). In particular, subtype A and CRF02_AG revealed improved outcomes (multivariable HR: 0.54 [0.29-0.98] and 0.39 [0.19-0.79], respectively).

Conclusions

Improved virological outcomes among patients infected with non-B subtypes invalidate concerns that these individuals are at disadvantage because drugs were designed mostly for subtype B infections.

Introduction

The HIV epidemic is characterized by a high genotypic diversity with multiple distinct viral subtypes and circulating recombinant forms (CRFs).[1] In North America, Europe and Australia, where most antiretroviral compounds were designed and initially tested, subtype B is predominant.[2] However, only approximately 10% of global HIV infections are caused by subtype B. The most prevalent subtype is C which occurs mainly in South and East Africa.[1]

With the introduction of combination antiretroviral therapy (cART) HIV/AIDS-related morbidity and mortality was markedly reduced,[3, 4] but concerns rose that antiviral susceptibility derived from studies with subtype B may not be applicable to non-B infections.[5] It was suggested that pretreatment genetic variation in the HIV reverse-transcriptase and protease among different subtypes may affect treatment response.[6] Studies in areas where non-B infections are predominant, mostly resource-limited settings, showed promising results, but these data can not be directly compared with data derived from resource-rich settings. To reduce biases, it is essential to perform inter-subtype comparisons in single settings.[7] A few studies were performed in Western countries analyzing the effect of viral subtype on treatment response.[8-15] However, all these studies had limitations and suffered either from a short follow-up time, a small sample size or the strong correlation of ethnicity and subtype.

We aimed to analyze short- and long-term effects of HIV subtype on the viral response after cART initiation in the Swiss HIV Cohort Study (SHCS). The SHCS provides the unique opportunity to study different subtypes in a single ethnic group, namely Caucasian. This is advantageous, because HIV subtype and ethnicity are strongly correlated and ethnicity is potentially associated with treatment response and with a different natural history of HIV.[16-20] Furthermore, it allows excluding potential bias due to different host genetic backgrounds.[21]

Methods

Study population

Data from the SHCS up to January 12, 2011 were included. The SHCS is a nationwide, multicenter, clinic-based cohort with continuous enrolment and semi-annual study visits [22]. The SHCS has been approved by ethical committees of all participating institutions, and written informed consent has been obtained from all participants. The present study was restricted to Caucasians with known HIV

subtype. Subtyping was based on sequences from the SHCS drug resistance database that are stored in SmartGene's (Zug, Switzerland) Integrated Database Network System (IDNS version 3.6.0).[23] Systematic retrospective sequencing was performed to obtain one sequence for each patient enrolled in the SHCS after 1995. Subtyping was performed using the REGA 2 System and if the results were inconclusive, we repeated subtyping with the Star analyzer (http://www.vgb.ucl.ac.uk/starn.shtml).[24] Sequences were excluded if the subtype remained unequivocal undetermined.

Study design

cART was defined as any antiretroviral therapy consisting of at least two different drug classes. Detection limits of HIV RNA assays changed in the course of time (<400 copies/mL before 1999, <50 copies/mL afterwards). Therefore, we performed two separate analyses with different definitions for viral suppression and virological failure. Analysis A included patients who initiated cART between January 1, 1996 and December 31, 2009. The definition of viral suppression was at least one viral load below the detection limit (<400 copies/mL) between day 90 and 365 after cART initiation. Virological failure was defined as: i) two consecutive viral loads >1000 copies/mL after previous suppression to <400 copies/mL on an uninterrupted treatment, or ii) one viral load >1000 copies/mL after previous suppression to <400 copies/mL followed by a treatment change or interruption, or iii) one viral load >1000 copies/mL after 180 days of treatment without previous suppression. If patients changed the cART regimen when viral load was suppressed, e.g. due to toxicity reasons, the definition of a virological failure for i) and ii) was adapted: Previous suppression to <400 copies/mL was not required during the new treatment. The analysis B included a subset of patients from analysis A. Analysis B was limited to treatment-naïve patients who started cART between January 1, 1999 and December 31, 2009. In 1999, all SHCS laboratories had changed their HIV RNA assays and achieved detection limits of 50 copies/mL. Viral load measurements with higher detection limits in this transition period occurred rarely and were excluded from analysis. The definition of viral suppression and virological failure was adapted in analysis B. Viral suppression was achieved when HIV RNA was <50 copies/mL and for the definition of virological failures the viral load limits in i), ii) and iii) were

changed: The lower limit was <50 copies/mL (instead of <400 copies/mL) and the upper limit >500 copies/mL (instead of >1000 copies/mL).

Statistical analysis

Baseline characteristics at cART initiation were analyzed with Fisher's exact test (categorical variables) and Wilcoxon rank-sum test (continuous variables). Baseline HIV RNA and CD4 cell counts were considered when measured within 180 days prior to cART initiation.

The short-term virological response (viral suppression) was analyzed using univariable and multivariable logistic regressions. Multivariable models were adjusted for sex, age, transmission category, baseline HIV RNA, baseline CD4 cell count, initial cART (unboosted PI, PI/r, NNRTI or other), calendar period (analysis A: 1996-1998, 1999-2003, 2004-2009; analysis B: 1999-2002, 2003-2006, 2007-2009), and previous treatment with mono/dual NRTI therapy (only analysis A). Continuous variables were categorized if likelihood-ratio tests indicated significant departures from linearity.

To study the long-term virological outcome, the virological failure rates were analyzed with Kaplan-Meier curves and log-rank tests. Additionally, univariable and multivariable Cox regression models were performed and adjusted for the same potential confounders described above. The proportional hazard assumption was checked with Schoenfeld residuals and by using graphical methods. While being pre-treated with mono/dual NRTI therapy in analysis A, did not satisfy the proportional hazard assumption, we stratified the Cox models for this variable. Co-linearity was checked and a variance inflation factor (VIF) <3 was tolerated for regression models. All analyses assumed intention to continue treatment and did not consider treatment changes after starting cART. Patient's follow-up was censored when the treatment was changed to a non-cART regimen. Periods of treatment interruptions were subtracted from the exposure time and viral loads measured off-treatment were not considered for analysis.

Self-reported adherence is measured since May 2003 in the SHCS and has been validated for treatment outcome.[25] We compared the lowest self-reported adherence between cART initiation and censoring or virological failure.

Statistical analyses were performed with Stata 11 SE (StataCorp, College Station, TX). All *P* values were two-sided and the level of significance was set at 0.05.

Results

Study population and baseline characteristics

Analysis A (cART start 1996-2009) included 4729 of 5268 patients (89.8%) with subtype B infections and 539 (10.2%) with non-B subtypes (table 1). The most common non-B subtypes were CRF02_AG (23.8%), A (23.4%), C (12.8%), CRF01_AE (12.6%) or other (27.5%). Most patients infected with "other" subtypes had a subtype F (29.1%, n=43), subtype G (28.4%, n=42), or subtype D infection (16.9%, n=25). CD4 cell count at baseline tended to be lower in patients infected with subtype B compared with non-B (median (IQR): 223 (106-357) cells/µl compared with 243 (134-366) cells/µl, *P*=0.088). The median $\log_{10}$ HIV RNA at baseline was similar between groups (subtype B: 4.7 [IQR: 3.9-5.2], non-B: 4.7 [3.9-5.3]).

In analysis B (cART start 1999-2009), 2166 of 2549 patients (85.0%) had subtype B infections and 383 non-B infections (15.0%). Most baseline characteristics were similar to analysis A (table 1).

First cART

In analysis A, 34.3% and 13.7% of patients infected with subtype B and non-B were pre-treated with mono/dual NRTIs, respectively (table 2). The median [IQR] year of cART initiation was earlier in patients infected with subtype B (1999 [1997-2004]) compared with non-B (2003 [1999-2007]), and they received more often unboosted PIs, 52.0% compared with 30.2% of non-B infections.

In analysis B, no difference between cART was present between groups (table 2). The median [IQR] year of cART start was similar: 2004 [2001-2007] and 2005 [2002-2007], respectively.

In both analyses, the most frequent NRTI combination was lamivudine and zidovudine. Efavirenz was the most common NNRTI and lopinavir the most frequently used PI/r. Patients with an unboosted PI received mostly nelfinavir or indinavir.

Patients of whom the treatment was not classified into the categories PI, PI/r or NNRTI had often combinations of PIs and NNRTIs (analysis A: 90/95, analysis B: 33/35).

Table 1. Patients characteristics at combination antiretroviral therapy (cART) initiation.

	Analysis A: cART start between 1996-2009								
Subtype	B n (%)	non-B n (%)	P^1	01_AE n (%)	02_AG n (%)	A n (%)	C n (%)	other n (%)	P^2
Sex			<0.001						<0.001
Male	3768 (79.7)	363 (67.3)		43 (63.2)	121 (94.5)	60 (47.6)	47 (67.1)	93 (62.4)	
Female	961 (20.3)	176 (32.6)		25 (36.8)	7 (5.5)	66 (52.4)	23 (32.9)	56 (37.6)	
Median age (IQR)	47(43-53)	50 (41-61)	<0.001	47.5 (42-59.5)	51.5 (43-58)	56 (45-67)	51 (45-61)	45.5 (39.5-59.5)	<0.001
Transmission category			<0.001						<0.001
HET	1082 (22.9)	388 (72.0)		58 (85.3)	84 (65.6)	101 (80.2)	50 (71.4)	96 (64.4)	
MSM	2253 (47.6)	91 (16.9)		7 (10.3)	36 (28.1)	10 (7.9)	10 (14.3)	28 (18.8)	
IDU	1250 (26.4)	37 (6.9)		1 (1.5)	4 (3.1)	8 (6.3)	5 (7.1)	20 (13.4)	
Other	144 (3.0)	23 (4.3)		2 (2.9)	4 (3.1)	7 (5.6)	5 (7.1)	5 (3.4)	
CDC stage			<0.001						<0.001
A	2536 (53.6)	366 (67.9)		47 (69.1)	93 (72.7)	79 (62.7)	50 (71.4)	98 (65.8)	
B	1255 (26.5)	101 (18.7)		16 (23.5)	13 (10.2)	25 (19.8)	14 (20.0)	34 (22.8)	
C	938 (19.8)	72 (13.4)		5 (7.3)	22 (17.2)	22 (17.5)	6 (8.6)	17 (11.4)	
CD4 count (cells/μL)			0.081						0.182
CD4 <200	1861 (44.4)	193 (40.2)		22 (37.9)	55 (48.3)	41 (36.9)	22 (34.9)	53 (39.3)	
CD4 ≥200	2330 (55.6)	287 (59.8)		36 (62.1)	59 (51.8)	70 (63.1)	41 (65.1)	82 (60.7)	
CD4 missing	538 (11.4)	59 (10.9)	0.830	10 (14.7)	14 (10.9)	15 (11.9)	7 (10.0)	14 (9.4)	0.910
HIV-1 RNA count (copies/mL)			0.884						0.193
<10,000	1258 (28.5)	141 (27.4)		14 (22.2)	23 (18.6)	43 (35.5)	19 (28.4)	43 (30.5)	
10,000-99,999	1583 (35.9)	187 (36.4)		25 (39.7)	47 (37.9)	44 (36.4)	27 (40.3)	45 (31.9)	
≥100,000	1571 (35.6)	186 (36.2)		24 (38.1)	54 (43.5)	34 (28.1)	21 (31.3)	53 (37.6)	
missing	317 (6.7)	25 (4.6)	0.065	5 (7.3)	4 (3.1)	5 (4.0)	3 (4.3)	8 (5.4)	0.415
	Analysis B: cART start between 1999-2009								
Sex			<0.001						<0.001
Male	1801 (83.2)	268 (70.0)		33 (61.1)	95 (97.9)	40 (48.8)	30 (68.2)	70 (66.0)	
Female	365 (16.9)	115 (30.0)		21 (38.9)	2 (2.1)	42 (51.2)	14 (31.8)	36 (34.0)	
Median age (IQR)	45 (39-51)	50 (40-61)	<0.001	46 (41-60)	52 (42-57)	55 (40-65)	51.5 (40-61)	46 (39-60)	<0.001
Transmission category			<0.001						<0.001
HET	533 (24.6)	265 (69.2)		44 (81.5)	63 (65.0)	62 (75.6)	30 (68.2)	66 (62.3)	
MSM	1147 (53.0)	76 (19.8)		7 (13.0)	32 (33.0)	6 (7.3)	7 (15.9)	24 (22.6)	
IDU	409 (18.9)	28 (7.3)		1 (1.9)	2 (2.1)	8 (9.8)	3 (6.8)	14 (13.2)	
Other	77 (3.5)	14 (3.7)		2 (3.7)	0 (0.0)	6 (7.3)	4 (9.1)	2 (1.9)	
CDC stage			0.044						0.050
A	1390 (64.2)	269 (70.2)		39 (72.2)	73 (75.3)	53 (64.6)	33 (75.0)	71 (67.0)	
B	422 (19.5)	68 (17.8)		12 (22.2)	8 (8.3)	16 (19.5)	8 (18.2)	24 (22.6)	
C	354 (16.3)	46 (12.0)		3 (5.6)	16 (16.5)	13 (15.9)	3 (6.8)	11 (10.4)	
CD4 count (cells/μL)			0.284						0.813
CD4 <200	819 (42.5)	134 (39.3)		17 (37.0)	37 (43.5)	27 (37.0)	16 (41.0)	37 (37.8)	
CD4 ≥200	1108 (57.5)	207 (60.7)		29 (63.0)	48 (56.5)	46 (63.0)	23 (59.0)	61 (62.2)	
CD4 missing	239 (11.0)	42 (11.0)	1.000	8 (14.8)	12 (12.4)	9 (11.0)	5 (11.4)	8 (7.5)	0.809
HIV-1 RNA count (copies/mL)			0.528						0.546
<10,000	450 (21.5)	89 (24.1)		11 (21.6)	15 (16.1)	25 (30.9)	10 (23.8)	28 (27.2)	
10,000-99,999	764 (36.5)	132 (35.7)		20 (39.2)	36 (38.7)	27 (33.3)	17 (40.5)	32 (31.1)	
≥100,000	882 (42.1)	149 (40.3)		20 (39.2)	42 (45.2)	29 (35.8)	15 (35.7)	43 (41.8)	
missing	70 (3.2)	13 (3.4)	0.876	3 (5.6)	4 (4.1)	1 (1.2)	2 (4.5)	3 (2.8)	0.774

[1] Fisher's exact test comparing subtype B and non-B infections, [2] Fisher's exact test comparing all particular subtypes
HET, heterosexual; MSM, men who have sex with men; IDU, injecting drug user; CDC, centers for disease control and prevention

Short-term virological outcome

In analysis A, 4433 of 4729 (93.7%) and 516 of 539 patients (95.7%) infected with subtype B and non-B had at least one viral load measured between day 90 and 365 after cART initiation (P=0.070). 3870 of 4433 (87.3%) and 481 of 516 (93.2%, P<0.001) achieved viral suppression. The probability to achieve viral suppression was higher in patients infected with non-B subtypes in the univariable logistic

regression model (odds ratio (OR): 2.0 [95% CI: 1.4-2.8]), but not in the multivariable model (OR: 1.2 [0.8-1.8]). Including patients without viral load measurement as failures did not alter conclusions. Results were similar in analysis B: 2076 of 2166 patients (95.8%) infected with subtype B and 375 of 285 (97.9%, *P*=0.060) with non-B subtypes had a viral load measured, of whom 1856 of 2076 (89.4%) and 338 of 375 patients (90.1%) achieved viral suppression (*P*=0.715). Compared with subtype B, non-B infected patients had a comparable probability to achieve viral suppression. The ORs in the univariable and multivariable models were 1.1 [0.8-1.6] and 1.0 [0.7-1.5], respectively. Considering missing values as treatment failures yielded similar results. Differentiating between the specific non-B subtypes did not alter conclusion. Compared with subtype B, probabilities to achieve viral suppression were not significantly different (data not shown).

Table 2. First combination antiretroviral therapy (cART)

	Analysis A: cART start between 1996-2009			Analysis B: cART start between 1999-2009		
Subtype	B n (%)	non-B n (%)	*P**	B n (%)	non-B n (%)	*P**
Year of cART initiation (analysis A | B)			<0.001			0.001
1996-1998 | 1999-2002	2198 (46.5)	113 (21.0)		617 (28.5)	75 (19.6)	
1999-2003 | 2003-2006	1164 (24.6)	165 (30.6)		660 (30.5)	138 (36.0)	
2004-2009 | 2007-2009	1367 (28.9)	261 (48.4)		889 (41.0)	170 (44.4)	
Pre-treated with mono/dual NRTIs	1624 (34.3)	74 (13.7)	<0.001	0 (0.0)	0 (0.0)	-
Treatment included:			<0.001			0.062
NNRTI	1035 (21.9)	177 (32.8)		863 (39.8)	157 (41.0)	
PI/r	1143 (24.2)	197 (36.5)		896 (41.4)	171 (44.6)	
PI	2458 (52.0)	163 (30.2)		373 (17.2)	54 (14.1)	
other	93 (2.0)	2 (0.4)		34 (1.6)	1 (0.3)	
NRTI backbone			<0.001			0.087
ETC TDF	644 (13.6)	114 (21.1)		598 (27.6)	108 (28.2)	
3TC AZT	1994 (42.2)	247 (45.8)		956 (44.1)	188 (49.1)	
3TC D4T	857 (18.1)	44 (8.2)		114 (5.3)	11 (2.9)	
D4T DDI	387 (8.2)	30 (5.6)		81 (3.7)	6 (1.6)	
3TC ABC	172 (3.6)	31 (5.8)		152 (7.0)	29 (7.6)	
3TC TDF	177 (3.7)	25 (4.6)		146 (6.7)	24 (6.3)	
Other NRTIs	498 (10.5)	48 (8.9)		119 (5.5)	17 (4.4)	
NNRTI			0.895			1.000
EFV	880 (85.5)	149 (84.2)		758 (87.8)	138 (87.9)	
NVP	148 (14.3)	27 (15.3)		103 (11.9)	19 (12.1)	
other NNRTI	7 (0.7)	1 (0.6)		2 (0.2)	0 (0.0)	
PI/r			<0.001			0.083
LPV	625 (54.7)	136 (69.0)		589 (65.7)	127 (74.3)	
ATV/r	224 (19.6)	37 (18.8)		195 (21.8)	33 (19.3)	
IDV/r	92 (8.1)	7 (3.6)		70 (7.8)	6 (3.5)	
Other PI/r	202 (17.7)	17 (8.6)		42 (4.7)	5 (2.9)	
Unboosted PI			<0.001			0.629
NFV	910 (37.0)	93 (57.1)		307 (82.3)	47 (87.0)	
IDV	949 (38.6)	42 (25.8)		31 (8.3)	5 (9.3)	
RTV	402 (16.4)	12 (7.4)		2 (0.5)	0 (0.0)	
Other PI	197 (8.0)	16 (9.8)		33 (1.1)	2 (3.7)	

*Fisher's exact test. NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PI/r, ritonavir boosted protease inhibitor; ETC, emtricitabine; TDF, tenofovir; 3TC, lamivudine; D4T, stavudine; DDI, didanosine; ABC, abacavir; EFV, efavirenz; NVP, nevirapine; LPV, lopinavir; ATV, atazanavir; IDV, indiniavir; RTV, ritonavir

Long-term virological outcome

In analysis A, 5268 patients contributed 29,446 person-years follow-up. The incidence of a virological failure was higher in patients infected with subtype B (4.3 [95% CI: 4.0-4.5] failures/100 person-years) compared with non-B (1.8 [1.4-2.4]). Incidences were smaller in analysis B, but patients infected with subtype B also had a higher incidence (2.6 [2.3-3.0] compared with 1.4 [0.9-2.1] failures/100 person-years; 2549 patients contributed 10,803 person-years follow-up).

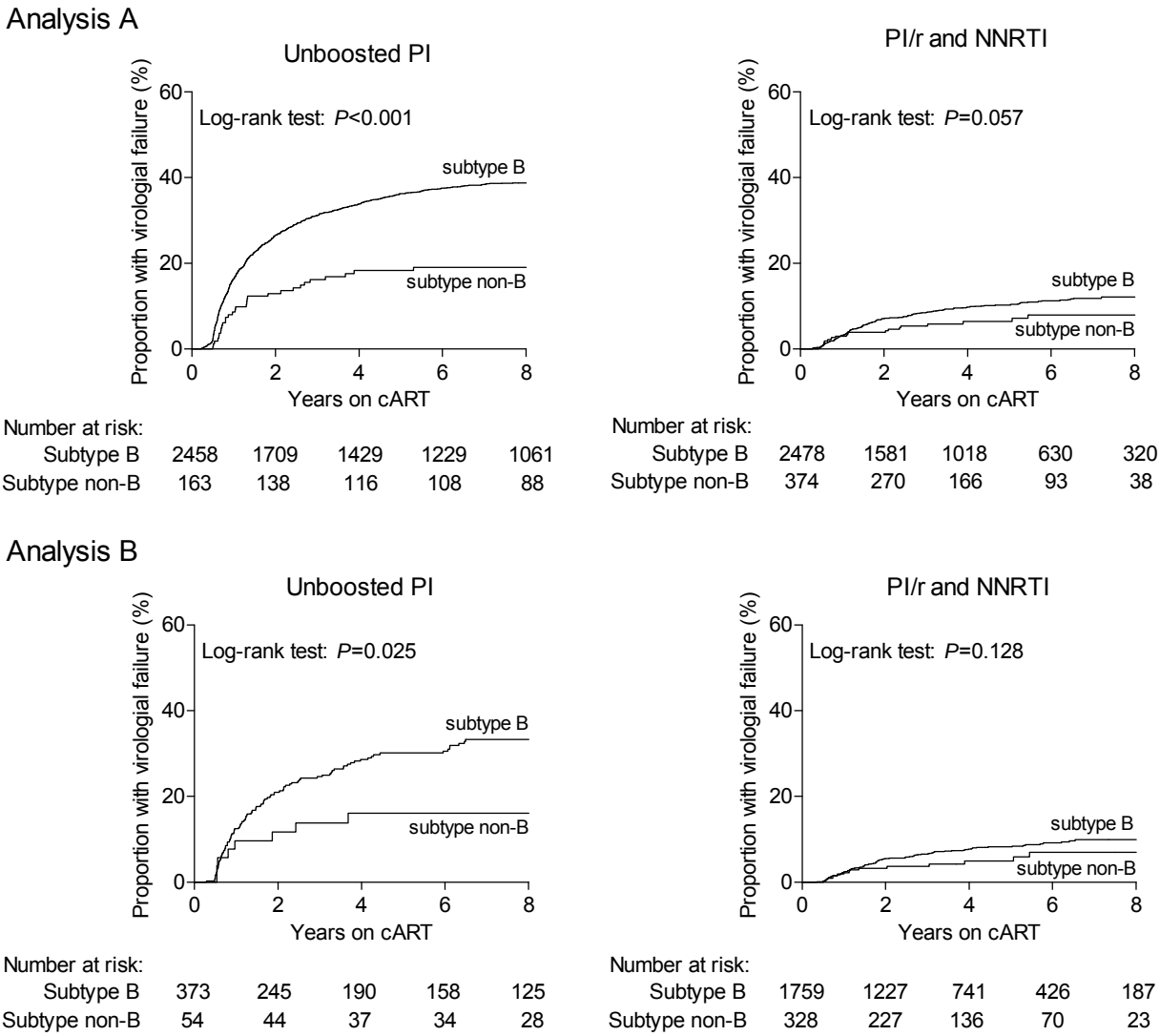

Figure 1. Kaplan-Meier curves differentiated by the first combination antiretroviral treatment (cART): unboosted protease inhibitor (PI), boosted PI (PI/r) or a nonnucleoside reverse transcriptase inhibitor (NNRTI). Analysis A and B included patients who started cART between 1996-2009 and 1999-2009, respectively.

Kaplan-Meier curves illustrate the time to virological failure differentiated by types of treatment (figure 1). As shown in Cox regression models, the probability to experience a virological failure was lower among patients infected with non-B

subtypes compared with subtype B (table 3). In analysis A, the univariable hazard ratio (HR) was 0.39 (95% CI: 0.30-0.52, <0.001) and the multivariable HR 0.68 (0.51-0.91, *P*=0.009). Analysis B retained similar results. The univariable HR was 0.54 (0.35-0.82, *P*=0.004) and the multivariable HR 0.63 (0.40-0.96, *P*=0.041). We additionally differentiated between the particular subtypes (figure 2). The multivariable Cox regression of analysis A showed that subtype A (*P*=0.042) and CRF01_AG (*P*=0.009) had significantly better long-term virological outcomes compared with patients infected with subtype B. No differences were found in analysis B, however sample sizes were small.

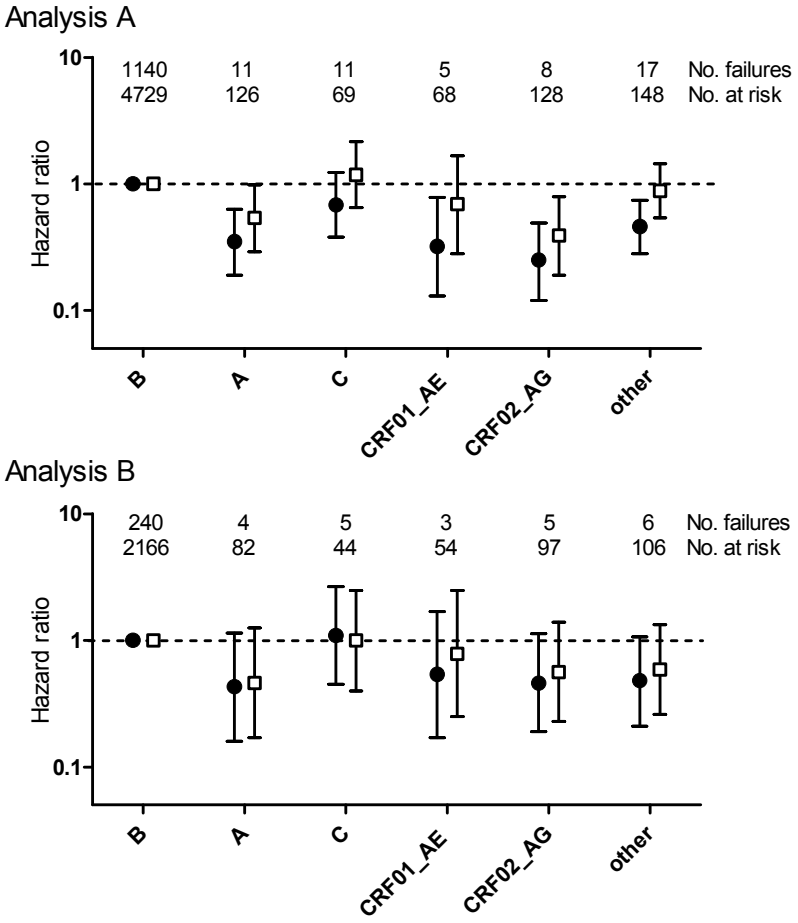

Figure 2. Univariable [●] and multivariable [□] Cox regression analyses comparing time to virological failure between patients infected with different HIV subtypes and circulating recombinant forms. Multivarible analyses are adjusted for age, sex, transmission category, first combination antiretroviral therapy (cART), baseline CD4 cells and HIV RNA. Analysis A is additionally stratified for previous mono/dual nucleoside reverse transcriptase inhibitor treatment. Hazard ratios below 1 indicate a better virological response compared with patients infected with subtype B. 95% confidence intervals are indicated.

Table 3. Cox regression models analyzing the time to virological failure.

Analysis A: cART initiation between 1996-2009							
	No. failures	No. at risk	% failures	Univariable HR (95% CI)	*P*	Multivariable* HR (95% CI)	*P*
Subtype							
B	1140	4729	24.11	Ref		Ref	
non-B	52	539	9.65	0.39 (0.30-0.52)	<0.001	0.68 (0.51-0.91)	0.009
Age (per 10				1.09 (1.03-1.15)	0.003	0.92 (0.86-0.99)	0.021
Sex							
Male	946	4131	22.90	Ref		Ref	
Female	246	1137	21.64	0.90 (0.78-1.03)	0.132	0.75 (0.64-0.87)	<0.001
Transmission					<0.001		0.355
MSM	496	2344	21.16	Ref		Ref	
HET	280	1470	19.05	0.86 (0.75-1.00)		1.09 (0.92-1.29)	
IDU	387	1287	30.07	1.43 (1.25-1.63)		1.11 (0.96-1.28)	
OTH	29	167	17.37	0.79 (0.54-1.15)		0.87 (0.60-1.27)	
CD4 cell count					<0.001		<0.001
CD4 <200	596	2054	29.02	Ref		Ref	
CD4 ≥200	415	2617	15.86	0.53 (0.47-0.60)		0.62 (0.54-0.71)	
CD4 missing	181	597	30.32	1.06 (0.90-1.25)		1.11 (0.79-1.55)	
HIV RNA					<0.001		<0.001
< 10,000	252	1399	18.01	Ref		Ref	
10,000-99,999	409	1770	23.11	1.36 (1.16-1.59)		1.65 (1.40-1.93)	
≥100,000	387	1757	22.03	1.24 (1.06-1.45)		1.59 (1.34-1.89)	
missing	144	342	42.11	2.39 (1.95-2.93)		1.32 (0.90-1.93)	
Treatment					<0.001		<0.001
PI	951	2621	36.28	Ref		Ref	
PI/r	126	1340	9.40	0.27 (0.23-0.33)		0.66 (0.54-0.82)	
NNRTI	97	1212	8.00	0.22 (0.18-0.27)		0.61 (0.47-0.79)	
Other	18	95	18.95	0.53 (0.33-0.84)		0.71 (0.44-1.15)	
cART initiation					<0.001		<0.001
1996-1998	899	2311	38.90	Ref		Ref	
1999-2003	239	1329	17.98	0.43 (0.37-0.49)		0.73 (0.60-0.87)	
2004-2009	54	1628	3.32	0.10 (0.07-0.13)		0.21 (0.15-0.30)	

While adherence to treatment is a potential bias, the self-reported adherence was similar between groups. In analysis A, 71.5% and 87.4% of patients had at least one documented self-reported adherence between cART initiation and the date of censoring or virological failure. Patients infected with subtype B and non-B had similar adherence: 45.7% and 49.9% never missed a dose, 27.7% and 28.2% missed a maximum of one dose per month, and 26.6% and 21.9% missed more than one dose per month (*P*=0.073). In analysis B, 87.6% (subtype B) and 93.5% (non-B) of patients had at least one reported adherence. Results were similar as in analysis A. Other factors potentially associated with a low adherence are high rates of treatment changes or an increased number of treatment interruptions. Both factors were similar between groups (data not shown).

Table 3. (continued)

Analysis B: cART initiation between 1999-2009							
	No. failures	No. at risk	% failures	Univariable HR (95% CI)	*P*	Multivariable* HR (95% CI)	*P*
Subtype							
B	240	2166	11.08	Ref		Ref	
non-B	23	383	6.01	0.54 (0.35-0.82)	0.004	0.63 (0.40-0.98)	0.041
Age (per 10				0.97 (0.86-1.09)	0.614	0.86 (0.75-0.99)	0.030
Sex							
Male	215	2069	10.39	Ref		Ref	
Female	48	480	10.00	0.96 (0.70-1.32)	0.808	0.76 (0.54-1.07)	0.110
Transmission					<0.001		0.099
MSM	101	1223	8.26	Ref		Ref	
HET	81	798	10.15	1.17 (0.88-1.57)		1.23 (0.89-1.72)	
IDU	72	437	16.48	2.05 (1.52-2.78)		1.52 (1.09-2.10)	
OTH	9	91	9.89	1.15 (0.58-2.28)		1.40 (0.70-2.80)	
CD4 cell count					0.002		0.055
<200	129	953	13.54	Ref		Ref	
≥200	100	1315	7.60	0.65 (0.50-0.84)		0.74 (0.56-0.97)	
missing	34	281	12.10	1.04 (0.71-1.52)		1.11 (0.66-1.84)	
HIV RNA					0.063		0.838
< 10,000	45	539	8.35	Ref		Ref	
10,000-99,999	85	896	9.49	1.09 (0.76-1.57)		1.02 (0.71-1.47)	
≥100,000	117	1031	11.35	1.20 (0.85-1.69)		1.13 (0.79-1.62)	
missing	16	83	19.28	2.27 (1.28-4.02)		1.21 (0.59-2.48)	
Treatment					<0.001		<0.001
PI	116	427	27.17	Ref		Ref	
PI/r	70	1067	6.56	0.27 (0.20-0.37)		0.51 (0.36-0.73)	
NNRTI	69	1020	6.76	0.25 (0.19-0.34)		0.46 (0.33-0.65)	
Other	8	35	22.86	0.82 (0.40-1.68)		0.94 (0.45-1.95)	
cART initiation					<0.001		<0.001
1999-2002	164	692	23.70	Ref		Ref	
2003-2006	65	798	8.15	0.33 (0.25-0.44)		0.48 (0.34-0.68)	
2007-2009	34	1059	3.21	0.19 (0.13-0.28)		0.30 (0.19-0.47)	

*Multivarible analyses are adjusted for age, sex, transmission category, first combination antiretroviral therapy (cART), baseline CD4 cells and HIV RNA. Analysis A is additionally stratified for previous mono/dual nucleoside reverse transcriptase inhibitor treatment. NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PI/r, ritonavir boosted protease inhibitor; HET, heterosexual; MSM, men who have sex with men; IDU, intravenous drug user; cART, combination antiretroviral treatment; HR, hazard ratio; CI, confidence interval

To assess the robustness of the finding that non-B subtypes have a better long-term virological outcome compared with subtype B, we performed several sensitivity analyses. In analysis A, results were similar if we excluded patients who were treated with mono/dual NRTIs prior to cART initiation (univariable HR: 0.46 [95% CI: 0.32-0.65], multivariable HR: was 0.58 [0.40-0.84]). If limiting analysis A and B to patients with known CD4 and RNA values at baseline, univariable HRs were 0.40 [0.29-0.53] and 0.56 [0.36-0.87], respectively. Multivariable HRs were 0.71 [0.52-0.97] and 0.66 [0.41-1.06], respectively. Results remained robust if we censored the follow-up when a treatment interruption occurred (analysis A, univariable HR: 0.38 [0.28-0.52]; multivariable HR: 0.62 [0.45-0.86]; analysis B: univariable HR: 0.62 [0.39-.99], multivariable HR: 0.69 [0.42-1.13]). The frequency of HIV RNA measurements was comparable between patients infected with subtype B and non-B, the median (IQR) days between measurements were 96 (79-119) and 92 (77-115) in analysis A, and

93 (79-117) and 91 (77-112) in analysis B, respectively. However, irregular or long durations without HIV RNA measurements might bias the results. Therefore we censored patients' follow-up if the time span between two HIV RNA measurements was longer than 180 days. Results remained robust (analysis A: univariable HR: 0.38 [0.28-0.50]; multivariable HR: 0.68 [0.50-0.93]); analysis B: univariable HR: 0.56 [0.37-0.85]; multivariable HR: 0.63 [0.40-0.98]). Moreover, the mode of transmission may be a critical issue. However, limiting the analysis to heterosexual patients did not alter conclusions (analysis A, univarable HR: 0.41 [0.29-0.59], multivariable HR: 0.61 [0.43-0.88]; analysis B: univariable HR: 0.41 [0.23-0.73], multivariable HR: 0.46 [0.26-0.83]). It was previously shown that transmitted antiretroviral resistance levels differ by subtype.[26] To assess whether our results could be due to differential baseline resistance, we performed a sensitivity analysis in a subset of patients who had a genotypic resistance performed prior to cART initiation (analysis A: n=3137 [59.6%], analysis B: n=2121 [83.2%]). The number of patients with transmitted mutations affecting the initial cART was slightly higher in the subtype B group (analysis A: 5.4%, analysis B: 4.3%) compared with non-B (each 2.4%). Point estimates of the multivariable Cox model for the effect of viral subtype (analysis A: 0.66 [0.40-1.10], analysis B: 0.80 [0.48-1.33]) were not substantially altered when adding information on transmitted resistance in multivariable models (analysis A: 0.68 [0.41-1.14], analysis B: 0.83 [0.50-1.39]).

Discussion

In the SHCS, we showed that Caucasians infected with HIV non –B subtypes had an improved virological success rate while treated with cART compared with patients infected with B subtype. In particular, subtype A and CRF01_AG infections were associated with an improved virological long-term response. The short-term virological response did not differ between subtypes.

In the last decade a vivid debate rose whether antiretroviral compounds are less active against non-B infections, since most antiretroviral drugs were designed to be used against subtype B infections.[7] Findings of this study indicate that these previously raised concerns are unwarranted.

This is the first study analyzing the impact of different HIV subtypes on treatment response in a single ethnicity, namely in Caucasians. Restricting the analysis to a single ethnic group is advantageous and avoids potential serious biases caused by

the association of ethnicity and subtype. Ethnic differences in host genetic factors influence the natural history of HIV, and the tolerability and potentially the efficacy of cART.[27] Furthermore, cultural differences between diverse ethnicities could influence virological outcome. The homogeneity of our cohort with regard to genetic and cultural backgrounds allows the assessment of the impact of viral subtypes on virological response independent of ethnical variability.[8, 16-19] Although, most patients infected with non-B subtypes are non-Caucasians, the question of susceptibility to cART among Caucasians infected with non-B subtypes becomes more and more important, since the prevalence of non-B infections is increasing in Western Countries.[26, 28]

Several *in vitro* studies were conducted to test the drug susceptibility of non-B subtypes. Overall, most non-B subtypes possessed similar susceptibilities to those of subtype B (reviewed in[6]). However, one study showed that CRF02_AG samples were more susceptible to nelfinavir and ritonavir.[29] In our study, the proportion of patients receiving these PIs was quite high which could partially explain our findings.

Our results differ from other previous published observational studies.[8-15, 18, 30] However, most of these studies were limited either by a small sample size, a short follow-up time, missing adherence data or the correlation of ethnicity and transmission category with the HIV subtype. So far, Geretti et al. published the largest study analyzing the effect of HIV subtype on cART response. They found no significant inter-subtype differences on long-term treatment response.[14] However, due to the strong correlation of HIV subtype with the ethnicity and the transmission group, they could not adjust their model for these two potential confounders.[16, 17, 21] In contrast, our study is unbiased by ethnicity and a sensitivity analysis clearly demonstrated that results remained robust if the analysis was limited to patients with heterosexual transmission. Furthermore, we used more restrictive criteria for virological failures. Geretti et al. did not ignore virological failures during treatment interruptions in the main analysis, only in a sensitivity analysis with highly reduced statistical power. However, both studies exhibit a rather small number of virological failures among patients infected with specific non-B subtypes. Contrary to Geretti et al., our study comprised a higher proportion of patients infected with subtype A, CRF01_AE and CRF02_AG and lower numbers of patients infected with subtype C and D.

Although, this is the largest study addressing the question of cART response among different subtypes in a single ethnic group, the sample sizes of some particular non-B subtypes were small and therefore confidence intervals of HRs remained wide. Larger cohort collaborations will be necessary to strengthen our findings. In our study, some baseline and treatment characteristics that are predictive for response to cART (e.g. treatment with unboosted PI) differed between patients infected with subtype B and non-B, especially in analysis A. However, results remained robust when adjusting the models for these factors. A sensitivity analysis excluded that our findings were substantially biased by differential resistance levels of transmitted viruses.

Previous concerns that antiretroviral treatment response might be hampered due to development and testing of antiretroviral compounds in resource-rich countries with high subtype B prevalence are not tenable anymore.

In conclusion, concerns that cART is less susceptible in non-B infections are unwarranted. In contrast, patients infected with particular non-B subtypes had a better long-term virological outcome in Switzerland.

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Conclusion

In the last few years several new antiretroviral drugs were licensed in Switzerland that are highly efficient against multi-drug resistant viruses, e.g. the new generation nonnucleoside reverse transcriptase inhibitor etravirine, the first integrase inhibitor raltegravir or the co-receptor antagonist maraviroc. The introduction of these drugs ushered a new era in HIV treatment. Its registration was probably the greatest breakthrough in HIV treatment since the introduction of the combination antiretroviral therapy. For the first time, highly treatment-experienced patients had the possibility to achieve long-lasting viral suppression with salvage regimens consisting of fewer pills and of drugs with less toxicity. Although the treatment options improved, it is highly relevant to closely monitor HIV drug resistance, especially in new salvage treatments, to early identify risk factors for the emergence of drug resistance and to ensure long-lasting treatment success. In resource-limited settings, the situation is different because the access to these new antiretroviral drugs is restricted. The quality of therapeutic monitoring mostly is very limited, viral load measurements are often not or less frequently performed, monitoring is often solely based on CD4+ cell counts, and HIV drug resistance tests are hardly ever performed in routine clinical care. Thus, the time on failing regimens can be very long and highly drug resistant strains can accumulate.

The Swiss HIV Cohort Study (SHCS) and the SHCS drug resistance database are characterized by a high data quality and data density. These are great tools to study the emergence, mechanisms and transmission of HIV drug resistance and help to improve, respectively optimize, antiretroviral therapy.

At a first glance, the six topics addressed may seem to be very diverse, but in fact they are all closely related and focus on important questions of today's HIV drug resistance research. The development of resistance mutations against new antiretroviral drugs is an important aspect. These drugs are often the last option for patients to achieve viral suppression and should therefore be administered in an optimal way to avoid the emergence of drug resistance. Even more, because the drug pipeline for new antiretroviral drugs developed by the pharmaceutical industry seems to begin to decline. In chapter 1 and 2, we studied aspects of HIV drug resistance to two new antiretroviral drugs, etravirine and raltegravir. Further, we

aimed to contribute to the knowledge of optimizing salvage treatments and studied in chapter 3 the impact of partially active or inactive nucleoside reverse transcriptase inhibitors in salvage treatment with raltegravir. Today very little is known about how to optimally treat patients with multi-drug resistant viruses. A combination of too many drugs may expose the patients unnecessarily to toxic drugs, whereas too few drugs can lead to early emergence of drug resistance and loss of the very important salvage components.

Another aspect of today's HIV drug resistance research is to monitor and study trends in resource-limited settings. Although, the SHCS drug resistance database only includes data from Switzerland, it is an invaluable tool to answer questions raised in resource-limited settings, because data from these countries are often insufficient and no comparable database exists. In chapter 4 and 5, we addressed some of these questions. The increasing prevalence of multi-nucleoside resistance mutations in resource-limited settings motivated us to identify risk factors for its emergence. In chapter 6, we also studied a subject that is mainly relevant in resource-limited settings where non-B subtypes occur very often. We studied the efficacy of antiretroviral drugs among non-B subtypes and found promising results. The long-term viral suppression was comparable, or even better, among patient infected with non-B subtypes compared with subtype B. The unique aspect of our study was that we had enough data to control for the ethnic background, meaning we were able to study different subtypes within the same host ethnicity. Thus, we were able to exclude host genetic effects to a large extent, an issue that is often totally neglected in this type of research.

To conclude, as long as HIV can not be cured, lifelong treatment with combination antiretroviral therapy will most likely be needed. Thus, HIV drug resistance will always emerge to a certain extent and potentially remain a severe obstacle to achieve longterm successful treatment results. The drug pipeline most likely will decline in the next few years and there will always be patients harboring multi-drug resistant viruses. Therefore, it is of high importance to focus in future research on optimization of salvage treatment strategies, thus the morbidity and mortality in these highly treatment-experienced patients can be reduced to a minimum. Further, it will be important to closely monitor drug resistance in resource-limited settings. Monitoring strategies in these countries should be improved to avoid the

accumulation of highly-drug resistant strains that can be transmitted. In these countries, high transmission rates of drug-resistant strains will have fatal consequences for newly infected patients, because the access to new drug classes is strongly limited due to lack of funds. Furthermore, it needs to be noted that ultimately emergence of drug resistance in resource-limited settings will also affect developed countries because of today's high global migration.

In conclusion, the field of HIV drug resistance is a dynamic topic and it is important to establish and maintain high-quality databases to monitor drug resistance and to be able to address new emerging research questions.

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